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Endodontic Post-Instrumentation Pain : A Bacteriological and Methodological Evaluation.

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ENDODONTIC POST-INSTRUMENTATION PAIN: A BACTERIOLOGICAL AND METHODOLOGICAL EVALUATION

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APPROVAL PAGE

Master of Dental Science Thesis

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A BACTERIOLOGICAL AND METHODOLOGICAL EVALUATION

Presented by

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INTRODUCTION

Instrumented Root Canals and Pain

After initial root canal instrumentation of asymptomatic teeth with necrotic pulps, patients sometimes experience severe pain. The reported frequencies of this pain range from 5 to 47% (Ingle and Zeldow, 1958; Seltzer *et al.*, 1961; Bartels *et al.*, 1968; Fox *et al.*, 1970; Clem, 1970; O' Keefe, 1976; Maddox *et al.*, 1977; Taintor and Ross, 1978; Soltanoff, 1978; Harrison *et al.*, 1981 and 1983; Pekruhn, 1981; Mulhern *et al.*, 1982; Balaban *et al.*, 1984; Mata *et al.*, 1985; Pisano *et al.*, 1985). According to a 1979 American Dental Association survey, over 17 million teeth in the United States undergo root canal therapy annually. Hence, if only 5% of these 17 million teeth were to exhibit such severe pain, severe post-operative pain constitutes a major clinical problem in root canal treatment. To date, there is no established method for predicting or preventing this severe pain.

Severe post-instrumentation pain (SPIP) is characteristically localized to an initially asymptomatic tooth which becomes percussion sensitive within 48 hours of initial root canal treatment. The pain is so severe that the patient almost invariably seeks emergency treatment.

SPIP patients may exhibit periapical swelling; however, this thesis will restrict attention to those without swelling in which painful symptoms of SPIP may not be a result of periapical abscess formation.

Dental pulpal necrosis (with or without swelling) and periapical abscess have been reported to be associated with mixed anaerobic infection (MacDonald *et al.*, 1957; Engström and Frostell, 1961; Sulitzeanu *et al.* 1964; Moore and Russell, 1972; Berg and Nord, 1973; Bergenholtz, 1974; Kantz and Henry, 1974; Wittgow and Sabiston, 1975; Keudell *et al.*, 1976; Sundqvist, 1976; Brook *et al.*, 1981; Byström and Sundqvist, 1981; Oguntebi *et al.*, 1982; Williams *et al.*, 1983; Yoshida *et al.*, 1987). However, no microbiological data from teeth with SPIP (without swelling) have been reported.

Etiologic factors hypothesized to be responsible for postoperative pain following root canal instrumentation are many: displacement of necrotic debris, microorganisms, or both through the apical foramen (Buckley, 1905); improper application or selection of intracanal irrigating solutions/medicaments (Harrison *et al.*, 1981 and 1983); incomplete or overinstrumentation of root canal(s), fracture of the tooth, or inflammatory exudation of the pulp or periapical region (Taintor and Ross, 1978); and/or endogenous release of pain-producing substances (Mergenhagen, 1972; Okuda *et al.*, 1978). A relationship between root canal microorganisms and the incidence of SPIP or of post-instrumentation pain, with or without swelling, however, has not been established.

The present study, using anaerobic and aerobic microbiological methods, attempted to establish whether there is a correlation between SPIP and the microbiological findings of root canal cultures obtained within 48 hours of initial instrumentation from SPIP patients without associated swelling. Within the limitations of detectability of root canal associated microorganisms, bacteria were found in 5 of 10 SPIP teeth. Furthermore, there was no correlation of pain with history of pulpal necrosis, radiographically evident periapical rarefaction, or overinstrumentation of root canals.

REVIEW OF THE LITERATURE

W.D. Miller (1894) theorized that pathologic conditions existing in pulps of carious teeth were related to metabolic by-products of bacteria. Onderdonk (1901) recommended, from what was then known about the causal relationship between bacteria in the root canal system and pulpal pathology, that root canal culturing should be performed during root canal treatment. Coolidge (1919) suggested root canals should be checked for "sterility" before obturation and Appleton (1932) argued that because bacteria do not always produce a putrid odor, confirmation of root canal "sterility" should be performed by bacteriological examination, namely, by sampling root canal contents.

Root canal culturing became a prominent procedure during the 1930s. It aided practitioners in associating the presence of bacteria detected within root canals with pulpal pathology, and it ultimately dispelled the focal infection theory whereby infected teeth were extracted to prevent systemic diseases (Sommer and Crowley, 1940; Reimann and Havens, 1940). Thus, cultures of these teeth after root canal instrumentation demonstrated that microorganisms could be eliminated from infected root canals, within detectable limits, without extraction of teeth (Cramer *et al.*, 1932; Coolidge, 1940).

Studies by Buchbinder (1941), Morse and Yates (1941), Oliet (1962), Winkler and van Amerongen (1959), and Zeldow and Ingle (1963) were in agreement about the importance of root canal culturing, but opinions differed about the necessity of anaerobic cultivation and the prognostic significance of positive cultures obtained at the time of obturation.

Seltzer *et al.* (1964) and Morse (1971) criticized aspects of earlier studies: aerobic incubation conditions for cultivation of anaerobes, inadequate

incubation periods (e.g., 48 hr), improper field isolation/disinfection procedures, inadequate sample size (e.g., only 1 paper cone used), and failure to ascertain longitudinally the significance of culture "reversals". These two papers, however, were based on conclusions primarily of anecdotal reports, not controlled studies.

Möller (1966), in a series of controlled studies, demonstrated that the improvement in detection of root canal microorganisms, which may have diverse nutritional requirements, required that the following conditions be achieved: a) the sampling fluid be nontoxic to bacteria, b) the sampling vehicle (e.g., paper cone) be effective for successful microbial retrieval, c) the transport medium be effective for sustaining viability until cultivation, and d) the culture media be supportive of the fastidious nutritional needs of the microorganisms in the sample.

Tables I:1 through I:4 summarize methodological aspects of eleven studies, published after Möller's 1966 report, which employ diverse root canal microbiological methods. As will be shown, few studies have improved our understanding of the microbiology of necrotic pulps beyond findings from Möller's work.

Table I:1 summarizes whether teeth were intact, whether and how the operative field was disinfected, whether the disinfectant was inactivated and whether efficacy of disinfection was validated. Studies which did not report whether the teeth were free from caries, restorations or exposure to the oral cavity are classified "NR", as indicated for Bence *et al.* (1973), Berg and Nord (1973), Griffee *et al.*, (1980) and Yoshida *et al.*, (1987).

TABLE I:1. Comparison of operative field disinfection procedures used for root canal culturing.*

STUDY	INTACT TEETH	DISINFECT FIELD	INACTIVATE DISINFECTION	VALIDATE DISINFECTION
Bence *et al.* (1973)	NR	3% H_2O_2 70% ETOH BACTINE	NO	NO
Berg & Nord (1973)	NR	35% H_2O_2 10% I_2 TINC	NO	NO
Bergenholtz (1974)	YES	35% H_2O_2 10% I_2 TINC	YES	YES
Kantz & Henry (1974)	YES	MERCOCRESOL	NO	NO
Wittgow & Sabiston (1975)	YES	70% ETOH 5% I_2 TINC	NO	YES
Keudell *et al.* (1976)	YES	NITROMERSOL	NO	NO
Kaufman & Henig (1976)	YES	NR	NO	NO
Sundqvist (1976)	YES	30% H_2O_2 5% I_2 TINC	YES	YES
Griffee *et al.* (1980)	NR	30% H_2O_2 2% I_2 TINC	YES	NO
Byström & Sundqvist (1981)	YES	10% H_2O_2 5% I_2 TINC	YES	YES
Yoshida *et al.* (1987)	NR	? I_2 TINC	NO	NO

*All 11 studies were reported after Möller's 1966 report.
ETOH = ethyl alcohol; H_2O_2 = hydrogen peroxide; I_2 tinc = iodine tincture; NR = not reported; NO = not done; and ? = unknown.

According to Sundqvist (1976), the study of intact teeth with intact pulp chambers (free from caries and/or restorations) is less likely to cause false positive samples by contaminating organisms from the oral cavity.

Most investigators disinfected the operative field with antiseptics before access was made into the pulp chamber. Hydrogen peroxide and iodine tincture, advocated by Möller (1966), were a frequent combination used by Berg and Nord (1973), Bergenholtz (1974), Sundqvist (1976), Griffee *et al.* (1980) and Byström and Sundqvist (1981), for this purpose. Kaufman and Henig (1976) did not describe a disinfection procedure and Yoshida *et al.* (1987) did not comment on the concentration of iodine tincture used. Bergenholtz (1974), Sundqvist (1976) and Byström and Sundqvist (1981) also inactivated the residual disinfectant in the field with 2% sodium thiosulfate and validated the adequacy of the disinfection procedure by rubbing a sterile paper cone against the lingual surface of the tooth and culturing it in thioglycollate broth. Wittgow and Sabiston (1975) did not comment on disinfectant inactivation but did check for field disinfection. Griffee *et al.* (1980) did not evaluate the disinfection procedure but did inactivate the disinfectant with sodium thiosulfate. These considerations perhaps reduced the frequency of isolation of microorganisms, if disinfectant remained, or increased the frequency of isolation of microorganisms, if disinfection was inadequate.

Table I:2 summarizes comparisons among root canal sampling fluids, sampling vehicles, transport media, and culture media used in the eleven studies.

TABLE I:2. Comparisons of sampling, transport, and cultivation media used for root canal culturing.

STUDY	SAMPLING FLUID	SAMPLING VEHICLE	TRANSPORT MEDIA	CULTURE MEDIA
Bence *et al.* (1973)	DIST H_2O	PAPER CONES/ REAMER	NONE	THIOGLYCOLLATE BROTH
Berg & Nord (1973)	CM BROTH	CHARCOAL PAPER CONES	BHIA ROLL TUBES	BHIA, HEMATIN & BLOOD AGAR; CMG BROTH
Bergen-holtz (1974)	VMG II	CHARCOAL PAPER CONES	VMG III	BLOOD & HUNTOON AGAR;THIO-GLYCOLLATE W/ 10% HORSE SERUM & POTATO
Kantz & Henry (1974)	RTF	PAPER CONE	RTF	THIOGLYCOLLATE, CM BROTH, MINIMAL AGAR & SPIROCHETE MEDIUM
Wittgow & Sabiston (1975)	NONE	PAPER CONE	NaCl	BHIA ROLL TUBES & BLOOD AGAR
Keudell *et al.* (1976)	NONE	PAPER CONE	PY	PY, PYG, BHIA, BLOOD AGAR
Kaufman & Henig (1976)	NONE	PAPER CONE	NONE	THIOGLYCOLLATE BROTH
Sundqvist (1976)	CMG BROTH	CHARCOAL PAPER CONES	BROTHS: PY, CMG MYCOPLASMA	BLOOD, MS, & ROGOSA AGAR
Griffee *et al.* (1980)	CMG BROTH	PAPER CONES	NONE	SCHAEDLER AGAR REDUCED THIOGLYCOLLATE BROTH
Byström & Sundqvist (1981)	NaCl	CHARCOAL PAPER CONES	PYG BROTH	PYG BROTH
Yoshida *et al.* (1987)	NONE	PAPER CONE/ REAMER	RTF	BLOOD AGAR

Möller's sampling fluid, Viability-preserving Medium Göthenburg I (VMG I), contained a combination of salts that was relatively nontoxic (a high concentration of cations, he noted was the likely cause of cell toxicity) to the bacteria he studied, as were his other media, VMG II, VMG III and VMG IV. Bence *et al.* (1973) used distilled water; Berg and Nord (1973) used chopped meat broth (CM), without glucose; Bergenholtz (1974) used VMG II; Kantz and Henry (1974) used reduced transport fluid (RTF); Wittgow and Sabiston (1975), Keudell *et al.* (1976), Kaufman and Henig (1976) and Yoshida *et al.* (1987) did not report using a sampling fluid; Sundqvist (1976) and Griffee *et al.* (1980) used chopped meat-glucose broth (CMG); and Byström and Sundqvist (1981) used saline as sampling fluid. Although no recent study has evaluated the toxicity of the sampling fluids listed in Table I:2, saline is reported to be toxic for some bacteria (Falk, 1920; Ballantyne, 1930; DeMello *et al.*, 1951).

For optimum recovery of microorganisms residing in the root canal system, Strindberg (1952) and Möller (1966) advocated the following technique: Using the largest coarse reamer which can be used to instrument the canal walls, the canal contents are agitated with a pumping motion (PMR method), thereby sampling all retrievable microorganisms from the canal system, including those from the dentinal tubules (Shovelton, 1964; Haapasalo and Ørstavik, 1987). The PMR method was also thought to detect microorganisms residing in the periapical tissue (Winkler *et al.*, 1972; Nair, 1987), granted that the apical foramen is penetrable by instrumentation (Engström, 1964) and that microorganisms are present (Shindell, 1961; Möller, 1966; Block *et al.*, 1976).

Most investigators used sterile paper cones as the sampling vehicle to absorb fluid from root canals (Table I:2). Berg and Nord (1973), Bergenholtz (1974), Sundqvist (1976), and Byström and Sundqvist (1981) used charcoal-impregnated paper cones, because the charcoal was thought to neutralize toxic substances in the sample.

Those who reported using only one paper cone may have reduced the ability of recovering a representative sample (Möller, 1966). In addition to paper cones, Bence *et al.* (1973) and Yoshida *et al.* (1987) placed root canal reamers (carrying necrotic debris resulting from filing the canal walls) into a transport medium. Because it was not reported whether sterile gloves were used during these procedures, sample contamination cannot be evaluated or excluded from these two reports.

During transfer of the sampling vehicles to transport media, Berg and Nord (1973), Kantz and Henry (1974), Wittgow and Sabiston (1975), Keudell *et al.* (1976), Sundqvist (1976) and Byström and Sundqvist (1981) flushed nitrogen gas over the tooth (Fulghum, 1971). This was thought to foster an anaerobic environment during the transfer procedure.

Another method thought to aid transportation of anaerobic microorganisms was described by Hungate (1950). The PRAS technique (prereduced, anaerobically sterilized) required culture media to be prereduced (by storage in an anaerobic environment) before use. McMinn and Crawford (1970) and Zielke *et al.* (1976 and 1979) confirmed the findings of Hungate's study. However, Bartlett *et al.* (1976), Loesche (1969) and Tally *et al.* (1975) maintained that anaerobes can tolerate limited exposure to air.

Transport media are sterile fluids used for transporting microbial samples from clinic to laboratory such that, ideally, growth of contained microorganisms is prohibited but viability is maintained. Their purpose is to allow subsequent inoculation of culture media with diverse microorganisms in the same proportions as they exist in the clinically infected site (Koneman *et al.*, 1983). Several media have been used for transport of root canal samples to the laboratory. Notably, some are really growth media (e.g., BHIA, PY, PYG and CMG*) whereas some others are salt solutions--either modified to foster reduced environmental conditions (e.g., VMG III or RTF) or not so modified (e.g., isotonic NaCl). VMG III is intermediate in composition, being a salt solution to which growth-promoting substances had been added (Gästrin *et al.*,1968).

Bence *et al.* (1973), Kaufman and Henig (1976) and Griffee *et al.* (1980) did not use a transport medium, but rather deposited paper cones directly into thioglycollate broth. According to Brewer (1940), thioglycollate medium had long been used for its presumed abilities to cultivate most anaerobes (attributed to its agar and sodium thioglycollate contents, the practice of boiling the medium to free it from oxygen before use, and its use in screw-capped test tubes). Nonetheless, fastidious, slow-growing anaerobes--if they could survive the "anaerobic" environment of this medium--would be susceptible to toxic products of facultative anaerobes, for example, streptococci, which readily grow in thioglycollate broth (Tanzer and McCabe, 1968).

Berg and Nord (1973) transported samples in BHIA roll tubes; Keudell *et al.* (1976) in PY medium; Sundqvist (1976) in PY, CMG and mycoplasma

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* BHIA= brain heart infusion agar; PY= peptone yeast; PYG= peptone-yeast-glucose; and CMG= chopped meat-glucose.

broths; and Byström and Sundqvist (1981) in PYG medium. In contrast to broth media, Wittgow and Sabiston (1975) transported samples in prereduced NaCl solution; Kantz and Henry (1974) and Yoshida *et al.* (1987) in RTF salt solutions; and Bergenholtz (1974) in VMG III (Table I:2).

As a result of studies published by Möller (1958, 1959, 1960 and 1966) and of emerging studies of periodontal and periapical abscess bacteriology (Newman and Socransky, 1977; Newman and Sims, 1979; Tanner *et al.*, 1979; Oguntebi *et al.*, 1982; Slots, 1986) there has grown an increasing realization of a prominent anaerobic flora associated with root canal, periapical, and periodontal lesions. As such, investigators increasingly used rich, complex media and anaerobic environments to facilitate recovery of fastidious anaerobes as well as facultative organisms.

Thus, in the eleven studies evaluated here (Table I:2) diverse liquid media, solid media, or both were used for cultivation of microorganisms. Bence *et al.* (1973), Kantz and Henry (1974), Kaufman and Henig (1976) and Griffee *et al.* (1980) used thioglycollate broth; Berg and Nord (1973) used CMG broth; Keudell *et al.* (1976) used PY and PYG broths; Byström and Sundqvist (1981) used PYG broth; and Bergenholtz (1974) used thioglycollate broth supplemented with 10% inactivated horse serum and a piece of potato. Additionally, the following solid media were used: blood agar and hematin agar (Berg and Nord, 1973), blood agar and supplemented Huntoon agar (Bergenholtz, 1974), a minimal agar and a spirochete medium (Kantz and Henry, 1974), blood agar (Wittgow and Sabiston, 1975; Keudell *et al.*, 1976; Sundqvist, 1976; Yoshida *et al.*, 1987), Mitis Salivarius (MS) and Rogosa SL

agars (Sundqvist, 1976), Schaedler agar (Griffee *et al.*, 1980), and BHIA in anaerobic roll tubes (Berg and Nord, 1973; Wittgow and Sabiston, 1975).

In Table I:3 (and Appendix F) the compositions of 3 transport media currently used for root canal cultures are detailed. MPRS is a salt solution containing L-cysteine (a reducing agent), resazurin (a redox indicator which is orange-brown above -42mV), and sodium metaphosphate (to aid dispersion of the sample).

TABLE I:3. Compositions of 3 transport media currently used for root canal culturing*.

MPRS		RTF		VMG III	
NaCl	2.25g	NaCl	0.90g	NaCl	1.00g
KCl	0.11g	K_2HPO_4	0.45g	KCl	0.42g
$CaCl_2$	0.63g	KH_2PO_4	0.45g	$CaCl_2$	0.24g
		$MgSO_4$	0.19g	$MgSO_4$	0.10g
		$(NH_4)_2SO_4$	0.90g		
		Na_2CO_3	0.40g		
		EDTA	0.1M		
SODIUM META-PHOSPHATE	10.00g			PHENYL-MERCURIC ACETATE	0.003g
				SODIUM GYLCERO-PHOSPHATE	10.00g
RESAZURIN	1µg	RESAZURIN	1µg	METHYLENE BLUE	0.002g
				AGAR (NOBLE)	2.00g
				BACTO-GELATIN	0.00g
				TRYPTOSE	0.50g
				THIOTONE	0.50g
L-CYSTEINE	0.50g	DTT	0.20g	L-CYSTEINE	0.50g
				THIOGLYCOLLIC ACID	0.50g
DEIONIZED H_2O	1000 ml	DEIONIZED H_2O	1000 ml	DEIONIZED H_2O	1000 ml

* MPRS: Modified prereduced 1/4-strength Ringer's solution, Manganiello *et al.* (1977).
RTF: Reduced transport fluid, Loesche *et al.* (1972).
VMG III:Viability-preserving medium III, Möller (1966).

RTF is also a salt solution containing dithiothreitol (DTT, a reducing agent), resazurin and ethylenediaminetetraacetic acid (EDTA, which is thought to disperse the sample by chelation of divalent cations). VMG III contains a combination of salts with added phenylmercuric acetate (a bacteriostatic agent); sodium glycerophosphate, tryptose, thiotone and gelatin (proteinaceous, growth-promoting substances); agar (thought to delay oxygenation of the medium), methylene blue (a redox indicator, blue above -230mV), and L-cysteine and thioglycollic acid (reducing agents).

MPRS was found to be effective in sampling and transport of anaerobes in deep periodontal pockets (Manganiello *et al.*, 1977) and therefore was the basis for its selection in this study. However, the original formula of MPRS, as described by Manganiello *et al.*, 1977, was modified in this study by the addition of 0.20g/L of DTT, to augment reducing agents contained in the medium.

Table I:4 summarizes the methods used to determine the presence of microorganisms in the transported sample, namely, whether stained or unstained smears were used, the periods of culture incubations (aerobic, anaerobic or both), and the frequency with which strict anaerobes were recovered among the total cultivable flora. Microorganisms which cannot be cultivated successfully may be detected in stained and unstained microsopic smears--either by brightfield, phase contrast, darkfield or Nomarski optics. For example, wetmount samples could be used to observe motility of microorganisms and to detect spirochetes--the latter are difficult to cultivate by artificial means (Rosebury and Reynolds, 1964; Socransky *et al.*, 1964).

TABLE I:4. Comparison of microscopic and cultivation procedures used for root canal culturing.

STUDY	DIRECT MICRO-SCOPY	DIRECT STAINED SMEARS	AEROBIC INCUBA-TION	ANAEROBIC INCUBA-TION	FREQUENCY STRICT ANAEROBES*
Bence *et al.* (1973)	NO	NO	5D	NO	?
Berg & Nord (1973)	NO	NO	?	?	79%
Bergenholtz (1974)	NO	NO	3D	6D	78%
Kantz & Henry (1974)	YES	YES	10D	7D	27%
Wittgow & Sabiston (1975)	YES	YES	5D	5D	75%
Keudell *et al.* (1976)	NO	NO	4HR	5D	64%
Kaufman & Henig (1976)	NO	NO	14D	NO	0%
Sundqvist (1976)	NO	NO	2D	7D	94%
Griffee *et al.* (1980)	NO	NO	7D	7D	?
Byström & Sunqvist (1981)	NO	NO	2D	10D	88%
Yoshida *et al.* (1987)	NO	NO	3D	7D	79%

* The values represent numbers of strict anaerobes recovered as a percentage of the total cultivable flora.
?= not reported.

Other investigators (Brown and Rudolph, 1957; Crawford and Shankle, 1961; Engström and Frostell, 1961; Feldman and Larje, 1966; Tanner *et al.*, 1979) have also argued for the use of direct microscopy in detecting microorganisms which are difficult to cultivate.

Of the eleven post-Möller studies, only Kantz and Henry (1974) and Wittgow and Sabiston (1975) used direct microscopic methods. Kantz and Henry (1974) recognized that direct microscopic smears revealed more organisms than could be recovered from non-selective agar plates and concluded that a large part of the microflora of necrotic pulp chambers could not be recovered by their methods.

Most of the investigators used both aerobic and anaerobic incubations for isolation of root canal bacteria. Berg and Nord (1973) did not stipulate the period of incubation and Kaufman and Henig (1976) did not incubate cultures anaerobically. Only Byström and Sundqvist (1981) incubated cultures anaerobically for more than 7 days. This was subsequently shown to be important for recovery of certain slow-growing bacteria, as for the recently reported *Bacteroides endodontalis* (van Steenbergen *et al.*, 1984; van Winkelhoff *et al.*, 1985).

The frequency of strict anaerobe isolation serves as an indicator of the effectiveness in anaerobic cultivation procedures. For example, Sundqvist (1976) had reported that 94% of the total cultivable flora recovered from intact teeth with necrotic pulps were strict anaerobes.

Berg and Nord (1973), Bergenholtz (1974), Wittgow and Sabiston (1975), Keudell *et al.* (1976), Byström and Sundqvist (1981) and Yoshida *et al.* (1987) had reported that 79, 78, 75, 64, 88, and 79%, respectively, of the total

cultivable flora were strictly anaerobic. Kaufman and Henig (1976) and Bence et al. (1973) did not report recovery of strict anaerobes probably because of the sole use of thioglycollate broth (which was neither prereduced nor incubated anaerobically). Griffee *et al.* (1980) did not comment on the frequency of isolation of strict anaerobes, in general, although they did recover black-pigmented *Bacteroides* species.

In view of the great diversity of methods used by Möller and the eleven subsequent investigations, it is not surprising that diverse microorganisms were found in necrotic pulps in those studies. Although the purpose of the present study (to be proposed and described below) was not to repeat the work of these twelve groups on necrotic pulp microbiology, the interested reader may examine the spectrum of microorganisms reported by those twelve studies (Appendix A).

OBJECTIVES OF THIS STUDY

General Objectives

From the literature on root canal, periapical abscess, and periodontal pocket microbiology, it appears that any study of root canal-associated microflora must provide adequate conditions for sampling, transporting, and cultivating anaerobic as well as aerobic/facultative microorganisms. In addition, direct microscopy is a useful adjunct for detection of microorganisms not recovered on selective and non-selective culture media.

Therefore, this study sought to evaluate microbial correlates of SPIP, using stringent anaerobic/aerobic bacteriological techniques, consistent with the implications of previous reports reviewed above.

The following questions were addressed.:

1. What is the probability of recovery of microorganisms from canals if the patient experiences SPIP?
2. Can microorganisms (if present) be detected if root canals had been instrumented/medicated?
3. Are there clinical and/or radiographic predictors of SPIP?

Specific Objectives

1. To determine the frequency of microbial recovery from root canals of symptomatic and asymptomatic teeth, sampled within 48 hours of initial root canal instrumentation.
2. To identify microorganisms recovered from root canals under study.
3. To examine the historical, clinical and radiographical correlates of SPIP.

MATERIALS AND METHODS

Subjects

Seven females and 3 males, 21 to 70 years of age, were studied microbiologically among the 38 subjects presenting to the University of Connecticut Health Center Dental Clinics and for whom endodontic treatment was performed either by residents or students under the supervision of the Department of Endodontics. All patients satisfied the following inclusionary criteria: presence of severe pain within 48 hours of initial root canal instrumentation, absence of facial or intraoral swelling, absence of periodontal pocket probing depths greater than 4 mm or pocket probings within 4 mm of the apex of the root, absence of systemic disease which could compromise host immune response, and absence of recent or current antibiotic treatment. Additionally, 4 subjects (negative controls) were studied for whom root canal instrumentation was performed and cultures obtained within 48 hours of treatment but who had no experience of SPIP. These cases were termed NPIP, no post-instrumentation pain. Informed consent was solicited from all participants in the study (see Appendix B).

Twenty-eight subjects were excluded from study according to the following criteria. (The number of subjects meeting each exclusionary criterion are given in parentheses.) The patient was a minor (1); the patient had a history of diabetes (1); the patient's tooth could not be isolated by rubber dam (7); the patient had received initial treatment by a private practitioner by indeterminate methods (11); the patient had swelling associated with the apex of the painful tooth (3); the patient's tooth had a temporary restoration with poor marginal

adaptation (1); the patient had a history of heart murmur (1); the patient had a history of liver disease (1); or the patient had a history of severe pain during initial treatment (2).

Collection of Root Canal Samples

Once teeth were deemed acceptable for study they were all handled as follows: local anesthesia administration by injection of Lidocaine with 1:100,000 (w/w) epinephrine, rubber dam isolation and cleansing of the field with 30% hydrogen peroxide for 1-2 minutes, and application of 5% iodine tincture for 3-5 minutes (Möller, 1966). To validate the disinfection procedure, the disinfectant residue in the field was inactivated with 10 ml of 2% sodium thiosulfate, and a medium-sized paper cone (Johnson and Johnson, E. Windsor, NJ) was rubbed against the lingual surface of the tooth (Sundqvist, 1976), placed in thioglycollate broth (BBL, Cockeysville, MD) and incubated for 7 days. Access to the root canal was then begun by superficial entry into the temporary restoration with a sterile bur, followed by reapplication of 5% iodine tincture. The disinfectant was again inactivated with thiosulfate and, using another sterile bur, the remaining temporary restoration was removed. The cotton pellet, if present in the canal, was removed and the canal(s) was (were) irrigated with 10 ml of the MPRS.

Then, using the PMR method, the canal contents were suspended in MPRS, all fluid was collected using medium-sized paper cones and immediately placed into a tightly sealed vial containing 5 ml of MPRS with no head space. The canal(s) was (were) flushed with 10 ml of 0.9% saline and, according to standard procedures in the Endodontics Clinic, the canal contents were again sampled by paper cones and placed in thioglycollate broth for 7

days incubation. Then, calcium hydroxide (prepared by mixing pharmaceutical $Ca(OH)_2$ with sterile saline) was placed into the canal(s) and the access opening sealed with IRM (Premier Dental Products, Philadelphia, PA).

Sample Transportation and Distribution

Samples contained in tightly sealed vials of MPRS were transported to the laboratory and placed into an anaerobic chamber (Coy Mfg., Ann Arbor, MI) containing an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. The contents of the vial were dispersed for 30 seconds by vortex mixing, aided by 1 mm diameter glass beads covering the bottom of each vial. These procedures were done within 30 minutes of canal sampling. Dispersion of contents was presumptively fostered by the presence of 1% sodium metaphosphate in the MPRS (Manganiello *et al.*, 1977), and reduced conditions were enhanced by addition of 0.02% DTT and 0.05% L-cysteine.

Dispersed cell suspensions were then handled in 3 ways: 1) streaked onto plates of appropriate selective, semi-selective, and non-selective agars; 2) smeared onto glass slides for Gram stain and brightfield examination; and 3) suspended in demineralized water for Nomarski interference microscopy for identification and rough quantitation of morphological types and detection of motility.

Cultivation of Root Canal Isolates

Various non-selective and differential agars (see Appendix D for formulae and conditions of use) were used for cultivation of root canal isolates: Brucella blood agar (BAK), supplemented with 5 µg/ml of hemin and 10 µg/ml of menadione (Phillips and Nash, 1986); BBE (Livingston *et al.*, 1978), without gentamicin, for esculin-positive bacteroides such as *Bacteroides fragilis* and

bile tolerant bacteria such as *Streptococcus faecalis* ; CFAT (Zylber and Jordan, 1978), for *Actinomyces* species; Clindamycin THFF-KNO_3 (Slee and Tanzer, 1978), for *Eikenella corrodens* ; EMB (Phillips and Nash, 1986), for gram-negative enteric bacilli (for potential sample contaminants); FEA, for *Fusobacterium necrophorum* and *Fusobacterium* species (Morgenstein *et al.*, 1981); GSTB (Tanzer *et al.*, 1984), for *mutans* streptococci; Rogosa SL agar (Rogosa, 1952), for *Lactobacillus* species; MS (Chapman, 1946), supplemented with potassium tellurite, for facultative streptococci; Mannitol-salt (Phillips and Nash, 1986), for *Staphylococcus* species (for potential sample contaminants); and TSBV, Slots *et al.* (1980), for *Actinobacillus actinomycetemcomitans.*

All agar plates to be used anaerobically were prereduced in the anaerobic chamber a minimum of 24 hours and were used within a week of preparation. Anaerobic incubations of agar plates continued for up to 14 days before cultures were considered negative and plates were discarded. In every case before use, 0.01 ml of MPRS was streaked onto BAK agar to confirm sterility of the medium.

Plates of BAK, BBE, Clindamycin THFF-KNO_3, FEA, Rogosa SL, and TSBV were routinely incubated in the anaerobic chamber and colonies were examined after 7 days. Plates of BAK, CFAT, EMB, GSTB, MS, and Mannitol-salt were incubated in candle extinction jars, which supplement air with approximately 4-5% carbon dioxide in partial replacement of oxygen, and examined after 2-4 days incubation to evaluate capnophilic organisms (Leadbetter *et al.*, 1979).

Representative colonies of all types of isolates were streaked for purity on BAK and, after 2-4 days, individual colonies were picked, suspended homogeneously in sterile demineralized water to preestablished turbidities, and used to inoculate the wells of the Rapid Strep system (API Analytab Products, Plainview, NY.; Applebaum *et al.*, 1984; Facklam *et al.,* 1984), for streptococci, and the An-Ident system (API Analytab Products, Plainview, NY) for strict anaerobes. Reference strains, some recommended by the manufacturers of these rapid identification strips, also were studied to maintain confidence in the results. In addition, confirmatory tests using more traditional identification methods were performed as further checks on reliability and accuracy of the rapid methods. These methods consisted of the ability of each isolate to ferment, in heart infusion broth (Difco, Detroit, MI), a 0.6% solution of the following: arabinose, cellobiose, fructose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. Also evaluated were catalase activity, esculin hydrolysis, gelatin liquefaction, indole production, litmus milk curd production and/or hydrolysis, nitrate reduction, starch hydrolysis, and urease activity.

All strains were subcultured every 7-10 days on BAK agar in the anaerobic chamber, and 5-day-old cultures were placed in GASPAK (BBL, Cockeysville, MD) jars, at room temperature, and were subcultured under anaerobic conditions every 30 days for maintenance.

RESULTS

Summary of Clinical and Bacteriological Findings

Ten teeth with SPIP were studied within 48 hours of initial root canal instrumentation, within the inclusionary criteria (detailed in "Materials and Methods"). Eight of 10 pulps had been necrotic, as judged by absence of bleeding, nonresponse to electric pulp test and thermal challenges.

From 4 of 10 root canals facultative and/or obligate anaerobes were isolated, yielding one (SPIP #1) to as many as 5 (SPIP #5) bacterial types. Suspected contamination from the oral flora, however, (evidenced by positive tooth surface culture) occurred during the culture procedure for one subject, SPIP #5. From one tooth (SPIP #7) gram-positive nonmotile rods were detected by a Gram stained smear and wetmount suspension, but were not successfully cultivated.

Of the 4 obligate anaerobes isolated from 10 SPIP teeth none were recovered from thioglycollate broth. In addition, from none of the cultures of the canal contents of the 10 SPIP teeth were spirochetes, yeasts, fungi or motile forms observed in Gram stains, wetmounts viewed by Nomarski optics, or on any agar plates.

Of the 4 pulps with no post-instrumentation pain (NPIP), 3 of 4 were necrotic, as judged by criteria described above. From none of these canals were bacteria detected/recovered.

A summary of the correlation of clinical and bacteriological findings is presented in Tables I:5 and I:6. For the interested reader, the individualized

data for each patient and the evidence leading to tentative identification of bacteria isolated from root canal cultures are given in Appendix C, Tables C:1 through C:11.

TABLE I:5. Summary of clinical and bacteriological findings in 10 SPIP and 4 NPIP subjects*.

SUBJECTS		GENDER /AGE	TOOTH ID #	PAR/ SIZE	WL FILE BEYOND APEX**	MICROFLORA ISOLATED
SPIP	1	F/50	1.4	YES/2	YES	*P. saccharolyticus*
	2	M/44	1.4	YES/4	NO	NONE
	3	F/22	1.5	NO	NO	NONE
	4	F/39	4.4	NO	NO	NONE
	5	F/31	4.6	YES/5	NO	*B. gingivalis* *F. nucleatum* *S. anginosus* (2) *S. sanguis* II
	6	F/22	4.6	YES/2	NO	*S. anginosus*
	7	M/66	1.3	YES/1	YES	NONE
	8	M/22	4.4	YES/7	NO	*B.gingivalis* *S. anginosus*
	9	F/27	2.3	YES/2	YES	NONE
	10	F/36	4.1	YES/10	YES	NONE
NPIP	1	M/37	4.1	YES/4	YES	NONE
	2	F/27	2.3	YES/2	YES	NONE
	3	M/23	1.1	YES/7	NO	NONE
	4	M/21	3.6	YES/3	NO	NONE

* SPIP= severe post-instrumentation pain. NPIP= no post-instrumentation pain.
** Tooth ID according to Federation Dentaire International (FDI) designation.
PAR= periapical radiolucency/ size in mm.(diameter).
WL= working length of root canal file placed beyond the apex (by radiographic criteria).

TABLE I:6. Key characteristics for identification of SPIP isolates*.

Organism	Gram Stain	Hem. on BAK	Ob/Fac Anaerobe	Fermen-tation	Biochem Rxns
P. saccharolyticus (SPIP 1IIA)	+	-	Ob	3,4,9	a,g
S. anginosus (SPIP 5IIA)	+	B	Fac	3,4,7,9, 13,15,16	e
B.gingivalis (SPIP 5IIB)	-	B	Ob	NONE	b,c,d,f
S. anginosus (SPIP 5IIC)	+	A	Fac	2,3,6,7, 9,13,15,16	b,e,h
F. nucleatum (SPIP 5IID)	-	-	Ob	**	
S. sanguis II (SPIP 5IIE)	+	A	Fac	2,3,6,7, 9,10,11,15	e,h
S. anginosus (SPIP 6IIA)	+	A	Fac	2,3,4,6,7, 9,10,13,15,16	b,e
S. anginosus (SPIP 8IIA)	+	A	Fac	2,3,4,6,7, 9,10,13,15,16	b,e,h
B. gingivalis (SPIP 8IIB)	-	B	Ob	None	b,c,d,f

* For Gram stain characteristics + = a positive reaction, - = a negative reaction; for hemolysis characteristics A = alpha-hemolysis, B = beta-hemolysis, and - = no detectable hemolysis; for anaerobic characteristics Ob = obligate anaerobe and Fac = facultative anaerobe.
The following numbers correspond with positive fermentation of carbohydrates: 1= arabinose, 2= cellobiose, 3= fructose, 4= glucose, 5= inositol, 6= lactose, 7= maltose, 8= mannitol, 9=mannose, 10= melibiose, 11= raffinose, 12= rhamnose, 13= salicin, 14= sorbitol, 15= sucrose, 16= trehalose. The following letters correspond with positive biochemical reactions: a= catalase activity, b= esculin hydrolysis, c= gelatin liquefaction, d= indole production, e= litmus milk curd production, f= litmus milk hydrolysis, g= nitrate reduction, h= starch hydrolysis.
** Although no fermentation or biochemical tests were performed on this isolate, (lost before these tests were performed) cellular, colonial and cultural characteristics were quite similar to reference strains of *F. nucleatum.* Thus, this isolate was presumably characterized as *F. nucleatum.*

DISCUSSION

During the period of this study 38 patients experienced postoperative discomfort following initial root canal instrumentation. The stringent inclusionary criteria used, however, limited study to only 10 of them. Admittedly, 10 SPIP samples are too few for statistical evaluation of some aspects of the findings. Nonetheless, findings of interest pertaining to the 10 SPIP and 4 NPIP teeth are discussed below.

One cannot simply correlate the incidence of SPIP with the presence of periapical radiolucency (8 of 10) or with initial pulpal necrosis before instrumentation (8 of 10) because 3 of 4 NPIP teeth were also initially necrotic and all had radiographically evident periapical rarefactions. Nonetheless, one cannot exclude the possibility that pulpal necrosis or periapical pathology at the time of patient presentation may predispose to the development of SPIP. To evaluate this particular question one would have to study the frequency of SPIP among pulps which were initially vital compared with pulps initially necrotic at the time of institution of endodontic therapy.

Bacteria were recovered from or observed in samples of root canals in 5 of 10 SPIP teeth; but from none of the 4 NPIP teeth could bacteria be recovered or observed. In one of the SPIP cases (#7), a single gram-positive nonmotile rod could be observed by Gram stain and Nomarski optics evaluation of smears, but could not be successfully cultivated. Some possible explanations for this finding are that dead/dying bacteria were detected on smears and thus not capable of growth on agar, that the BAK could not support growth of these bacteria, and/or that too few bacteria were present in the culture sample to be evident after culture dilution. In another of these cases (SPIP #5) culture of the

root canal yielded 5 individual isolates, two of which (*S. anginosus* and *S. sanguis* II) were also recovered from the tooth surface during evaluation of surface disinfection. The two *S. anginosus* strains recovered from root canals, however, were distinct: One was beta-hemolytic, did not hydrolyze esculin and fermented neither starch nor lactose, whereas the other was alpha-hemolytic, hydrolyzed esculin and fermented both starch and lactose. In addition, the latter isolate was apparently also recovered from the outside of the tooth, whereas the former isolate was recovered from the root canal only.

The *S. sanguis* II strains recovered from outside and inside the tooth, however, were not distinguishable. It is, perhaps, of further interest that this tooth had been restored by silver amalgam and that access preparation for root canal therapy had been made through the silver amalgam restoration, a procedure suggested by others to invite pulpal contamination by oral flora (Sundqvist, 1976). In no other case in this series had access been made through an existing restoration or evidence of tooth surface contamination.Two other SPIP teeth yielded recoveries of *S. anginosus*, one as a pure culture (SPIP #6) and one in mixed culture with *B. gingivalis* (SPIP #8). *B. gingivalis*, as well as *F. nucleatum*, had also been isolated from SPIP #5 from which *S. anginosus* and *S. sanguis* II had also been recovered, as described above. One case (SPIP #1) gave recovery of a pure culture of *P. saccharolyticus* and peptococci have recently been reported in necrotic pulps of symptomatic teeth (Yoshida *et al.*, 1987).

The identities of the anaerobes were not confirmed by gas-liquid chromatography. Furthermore, obligate anaerobes could not be identified with certainty by the AN-IDENT system alone--including the identification of

recommended ATCC type strains. Thus, additional tests were necessary for confirmation of identification. More clear results were obtained, however, using the Rapid Strep system for identification of streptococcal species, as confirmed by additional tests.

Previous literature suggests that periapical abscesses of humans yield recoveries of mixed rather than pure cultures (Brook *et al.*, 1981; Oguntebi *et al.*, 1982; Williams *et al.*, 1983). Notably, certain bacterial types are frequently found together, for example, a bacteroid with a streptococcus or a fusobacterium with a streptococcus. In general, strict anaerobes and facultative anaerobes are found concurrently (Sundqvist, 1976; Byström and Sundqvist, 1981; Oguntebi *et al.*, 1982; Yoshida *et al.*, 1987), but occasionally only facultative or only anaerobic mixtures are found (Sundqvist, 1976; Oguntebi *et al.*, 1982). This was consistent with those SPIP cases in which either strict anaerobes, facultative anaerobes or both were isolated.

The taxonomy of *S. anginosus* has been in flux, such that strains with similar characteristic have sometimes been called either *S. milleri*, *S. intermedius* or *S. constellatus*, thus confusing the literature. Nonetheless, due to reported greater similarities than differences among their DNA base pairs it has been proposed that they all be termed *S. anginosus* (Coykendall *et al.*, 1987), as done here.

Negative recoveries of bacteria and negative Gram stains and Nomarski examinations were obtained from post-instrumentation root canals of five subjects with SPIP and all four subjects with NPIP. This could have reflected the true absence of microorganisms from these canals, failure to obtain a representative sample of the root canal system, failure of the transport medium,

or failure to successfully grow microorganisms on the diverse agar media. Attempts were made to minimize these possibilities, as follows: 1) by use of the PMR method to agitate canal contents and debride canal walls; 2) by use of MPRS, which had been described as minimally toxic to anaerobic microorganisms (Manganiello *et al.*, 1977), as sampling fluid together with two or more paper cones per canal; 3) by use of the same medium to transport samples to the laboratory, taking further advantage of its putative nontoxicity, the documented survival of periodontal anaerobes in high numbers in it (Manganiello *et al.*, 1977), and by addition of DTT to further enhance reduced conditions in the medium; 4) by cultivation of transported samples within 30 minutes in an anaerobic environment using a variety of non-selective, semi-selective and selective agars, appropriately incubated; and 5) by use of direct microscopic examination of transported samples by Gram stain and Nomarski optics to examine for noncultivable bacteria. It is highly unlikely, therefore, that presence of microorganisms in samples would have been totally missed. Nonetheless, caution must be expressed because one sample (SPIP #7) revealed microorganisms which proved to be initially dead or uncultivable.

Thioglycollate cultures of root canals of all SPIP cases taken immediately following MPRS samplings recovered the facultative organisms revealed by MPRS but failed to recover the strict anaerobes, even though thioglycollate, historically, has presumably been used for detection of anaerobes (Brewer, 1940).

It is conceivable that antimicrobials [NaOCl, I_2KI, and $Ca(OH)_2$] placed in canals at the time of initial instrumentation or at canal closure could have not only influenced the symptomatic state of the treated tooth but the recoveries of

possible microorganisms during the 24 to 48 hours subsequent to instrumentation (Spangberg, 1982). To inactivate antimicrobial agents remaining in the canals during the culture procedure, the canals were flushed with 10 ml of MPRS. Further inactivation of iodine potassium-iodide with 2% sodium thiosulfate at the time of root canal sampling of SPIP and NPIP patients, advocated by Möller (1966), was not performed due to suspected toxicity of sodium thiosulfate for some bacteria (Gross and Huff, 1973).

It is generally believed that instrumentation beyond the root canal system will cause clinical exacerbation, frequently manifested as pain (Sundqvist, 1976; Balaban *et al.*, 1984). Because case records were kept of filing beyond the tooth apex (4 of 10 SPIP, 2 of 4 NPIP cases), it was possible to determine the lack of apparent association between this phenomenon and of individual cases with post-instrumentation pain.

Overall, there was a sense that the weakest component among the root canal cultivation procedures was likely to be the transport medium, MPRS. To evaluate that question, a series of experiments was performed which will be described in Study II.

SUMMARY

Ten patients having SPIP within 48 hours of initial root canal instrumentation were studied to evaluate the presence or absence of microorganisms in root canals of these teeth. There was no correlation of pain with history of pulpal necrosis, radiographically evident periapical rarefaction, or overinstrumentation of canals.

Within limitations of the techniques used for bacterial recoveries, strict and facultative anaerobes were isolated, streptococci most frequently. Certain types of anaerobic bacteria previously reported to have been recovered from necrotic pulps were not detected; neither were spirochetes detected. However, other anaerobic bacteria were recovered.

Six subjects with severe pain (SPIP) had no recoverable bacteria from the root canal(s) of their painful teeth, whereas 4 had as few as one or as many as 5 different types isolated. There was concern that false positive samples could have occurred if the tooth contained caries or restoration, and there was concern that false negative samples could have occurred if the transport medium, MPRS, was unreliable.

Four patients without pain (NPIP) within 48 hours of initial root canal instrumentation had no detectable bacteria from their root canals.

CONCLUSIONS

Within the ability of the presently used methods to detect microorganisms in root canals with infected pulps that have been instrumented/medicated, the following can be concluded:

1. There is a notable association of *Streptococcus anginosus* (three times), *Bacteroides gingivalis* (twice), and *Fusobacterium nucleatum*, *S. sanguis* II, and *P. saccharolyticus* (each once) with SPIP. However, because of the limited number of subjects meeting the rigorous criteria for inclusion, additional studies are needed to better define the microbiological correlates of post-instrumentation pain.

2. Additional studies are needed in which intact teeth with infected pulps will be cultured to establish baseline data on their pulpal flora. Then, if SPIP occurs, subsequent cultures should be performed. This will aid in identifying specific bacterial risk factors which may determine development of SPIP. Such studies have been proposed.

Study II: Evaluation of Transport Media for Root Canal Bacteria

INTRODUCTION

Efficacy of Root Canal Transport Media

The observations of Study I, of negative recoveries from some SPIP samples and positive recoveries from others--but of only certain types of flora, led to the development of an *in vitro* model by which some parameters of root canal culturing could be evaluated further. More important, because there was concern about the effectiveness of MPRS, studies were designed (as will be detailed below) to compare it with other media currently used for transporting oral cultures suspected of containing fastidious anaerobes.

REVIEW OF THE LITERATURE

Stuart (1954 and 1956) evaluated various media for the transportation of gonococci from genital lesions from the clinical setting to the laboratory. Möller (1966) evaluated his VMG media and Stuart's medium for the transportation of bacteria recovered from root canals to the laboratory. Gästrin *et al.* (1968) evaluated Stuart's, a modified Stuart's (SBL), and Möller's VMG IV media for the transportation of clinical isolates to the laboratory. Both Syed *et al.* (1972) and Rundell *et al.* (1973) evaluated RTF, SBL and VMG II media for the transportation of facultative bacteria, especially streptococci and strict anaerobes recovered from plaque, to the laboratory. These studies, however, evaluated the efficacy of transport of large inocula. Because transport of root canal contents after canal instrumentation may contain few microorganisms (Engström, 1964), efficient transport of few cells needs to be studied.

Möller (1966) in developing his transport media (VMG II, III and IV) proposed that these media must be able to preserve viability of even few bacteria which may exist in the root canal system. This requires, however, adding substances to the medium that may either inhibit growth of some bacteria or promote growth of others. The problem of growth of some bacteria in Möller's VMG media was demonstrated by Gästrin *et al.* (1972), Jordan *et al.* (1968) and Syed and Loesche (1972).

Manganiello *et al.* (1977) determined that anaerobes located in periodontal pockets could be recovered in a simple salt solution, MPRS. van Winkelhoff *et al.* (1985) successfully cultivated *Bacteroides endodontalis*, a fastidious anaerobe, using MPRS as transport medium.

Thus, MPRS appeared to have at least some utility as a transport medium for root canal cultures. RTF and VMG III have also been shown to be effective for supporting facultative and anaerobic bacteria until cultivation (Syed *et al.*, 1972; Bergenholtz, 1974; Yoshida *et al.*, 1987). Nevertheless, no study has demonstrated whether MPRS, RTF or VMG III has the ability to preserve the viability of a low number of various bacterial types stored in that medium.

OBJECTIVES OF THIS STUDY

General Objectives

The reviewed literature and the results of Study I (above) led to *in vitro* - experiments comparing the efficacy of MPRS and RTF and, subsequently, VMG III. Known numbers of diverse facultative and strict anaerobes, many of which have been encountered in root canals, were selected for study. We hypothesized that MPRS, RTF and VMG III are equally good transport media.

Specific Objectives

1. To make quantitative *in vitro* comparisons of MPRS, RTF and VMG III, evaluating expected versus observed CFU recoveries of a spectrum of strict and facultative anaerobic bacteria for up to 24 hours storage time, and evaluating the viability-preserving and growth-inhibiting abilities of each transport medium.
2. To make *in vivo* comparisons between MPRS and RTF, sampling from root canals of necrotic pulps.

MATERIALS AND METHODS

In Vitro Evaluation of MPRS, RTF and VMG III

Bacterial strains (Table II:1), representative of some of the types reported to have been isolated from infected pulps and strains likely to be among the most difficult to transport due to their fastidious and generally anaerobic requirements, were used to compare quantitative recoveries of colony forming units (CFU) from either modified 1/4-strength prereduced Ringer's solution (MPRS*) or reduced transport fluid (RTF**) for up to 24 hours storage time at room temperature.

Table II:1. List and source of 26 bacterial strains used for transport media evaluations.

GENUS/SPECIES	STRAIN	SOURCE
Actinobacillus actinomycetemcomitans	ATCC 33384	ATCC
Actinomyces naeslundii	ATCC 12104	TANZER
Bacteroides asaccharolyticus	ATCC 25260	ATCC
Bacteroides asaccharolyticus	ATCC 27067	ATCC
Bacteroides endodontalis	BNIIA-F	MAYRAND
Bacteroides fragilis	ATCC 25285	ATCC
Bacteroides gingivalis	ATCC 33277	KIEL
Bacteroides gingivalis	W50	MAYRAND
Bacteroides gingivalis	W83	MAYRAND
Bacteroides gingivalis	6126	MAYRAND
Bacteroides intermedius	ATCC 25611	KIEL
Bacteroides intermedius	5W2	MAYRAND
Bacteroides melaninogenicus	ATCC 25845	ATCC
Bacteroides oralis	ATCC 33269	ATCC
Capnocytophaga ochracea	ATCC 27872	ATCC
Eubacterium nodatum	ATCC 33099	ATCC
Fusobacterium nucleatum	JP2	SOCRANSKY
Fusobacterium nucleatum	JN9	SOCRANSKY
Fusobacterium nucleatum	JG16	SOCRANSKY
Peptococcus asaccharolyticus	ATCC 29743	ATCC
Peptococcus saccharolyticus	ATCC 14953	ATCC
Peptococcus saccharolyticus	SPIP 1IIA	CARRINGTON
Peptostreptococcus anaerobius	ATCC 27337	ATCC
Streptococcus anginosus	SPIP 6IIA	CARRINGTON
Streptococcus faecium	ATCC 9790	TANZER
Streptococcus sanguis II	SPIP 5IIE	CARRINGTON

* Without 0.0001% Resazurin

** Without 0.0001% Resazurin and 0.1M EDTA.

Then, using most of the same strains, RTF and VMG III were analogously compared (see below for modification of storage temperature). Each experiment was performed at least twice to establish consistency of the data generated.

Reference strains were first Gram stained and identities confirmed by evaluation of cellular, colonial, cultural, and biochemical characteristics. The AN-IDENT system for anaerobes or the Rapid Strep system for streptococcal species also were used to establish identification, as needed.

The paper cone transfer (PCT) method used in this study was as follows: Each strain was cultured on the non-selective Brucella blood agar (BAK), supplemented with 5 μg/ml of hemin and 10 μg/ml of menadione. When colonies reached 0.5 to 1 mm in diameter they were suspended in prereduced saline to approximately 3 x 10^8 organisms/ml. After serial dilution in saline (to about 10^5 organisms/ml) 10 μl were deposited from a micropipette onto each of 2 medium-sized sterile paper cones (Johnson and Johnson, E. Windsor, NJ), and 2 such cones were then placed into four replicate vials each containing a total capacity of 5 ml of MPRS or RTF (Fig. 1).

The decision to use four replicate vials rather than one vial four times was based upon a preliminary study in which samples of *S. faecium* (ATCC 9790) were deposited on paper cones, placed in transport medium, and vortex mixed at 0, 1, 4 and 24 hours, immediately prior to plating on BAK agar.

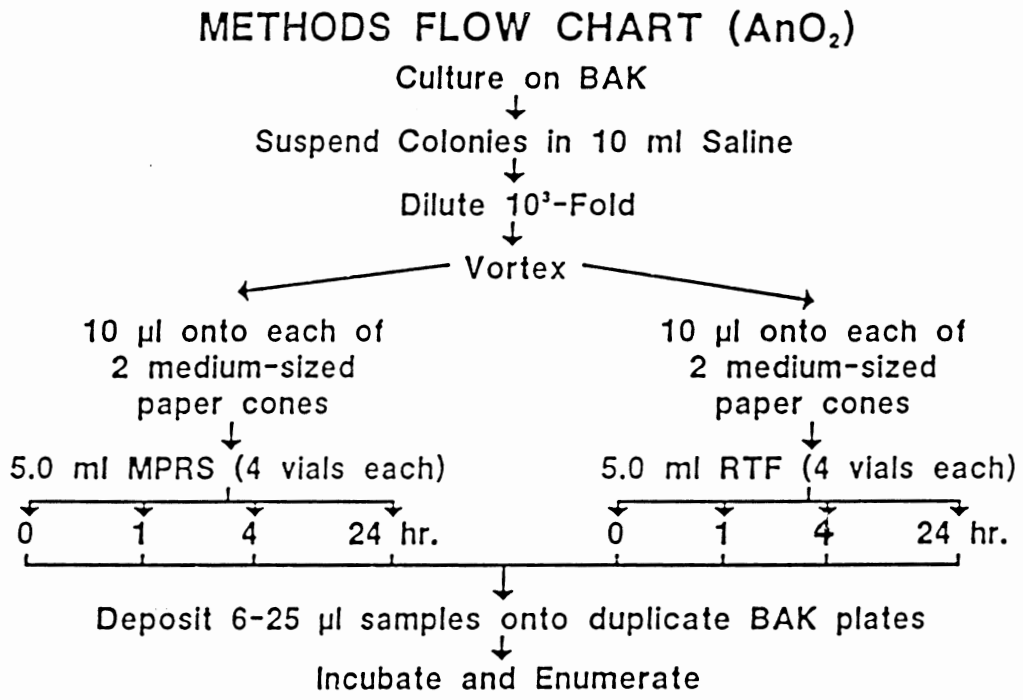

Figure 1. Diagram of paper cone transport (PCT) method used for *in vitro* transport media evaluations.

Because CFU counts dramatically increased with time in this experiment, there was concern that either the cells were dividing, the chains were breaking due to vortex mixing with beads, autolysis of septa was occurring, or that cells were not immediately being released from the paper surfaces. For this reason, as a routine, 4 replicate samples were placed in individual transport medium vials and were vortex mixed only once at the time of culturing on BAK. The placement of paper cones was alternated between the two transport media so as to reduce possible bias of the sequence. Thus, a first and third paper cone were placed in MPRS and a second and fourth paper cone in RTF for one storage time. Then, a first and third paper cone were placed in RTF and a second and fourth placed in MPRS for the next storage time, and so forth.

After vortex mixing for 30 seconds, 6-25 µl samples from each vial, at specified times, were deposited onto duplicate BAK plates using a technique similar to that of Westergren and Krasse (1981). The expected final dilution was about 30 CFU/25 µl. This value was chosen so as to avoid confluent growth of colonies on the agar plates which would have precluded quantitative evaluation. For each strain the quantitative recoveries from the transport media were evaluated at 0, 1, 4, and 24 hours of storage time (outside of the anaerobic chamber at room temperature, albeit in sealed vials).

After each 0-hour sample was deposited onto BAK medium, as described above, 3-25 µl samples of the saline-suspended bacteria were deposited directly onto BAK agar to check cell viability in each suspension. If there were no colonies grown from the saline suspensions, no recoveries could have been expected from transport media and results could not be ascribed to transport medium failure.

In the course of these experiments, it was observed that CFU recoveries in RTF were superior to those in MPRS, as will be detailed below. Accordingly, the study was refocused upon a comparison between RTF and VMG III. Thus, in a similar experiment RTF was compared with VMG III with the only difference being that vials of RTF and VMG III were placed in a 37° C water bath 15 minutes before depositing the 1-, 4- and 24-hour samples. This was done to reduce the viscosity of the VMG III which becomes semisolid at room temperature. The RTF also was warmed to maintain comparability of the experimental methods. All other procedures and incubations, however, were performed in the anaerobic chamber at 35° C, in an identical fashion to that described above.

There was concern that some microorganisms could be adversely affected by exposure to prereduced saline. The effect of water, saline, MPRS, RTF or VMG III on cell viability was, therefore, determined after 30 minute exposure to that diluent. All 26 bacterial strains were studied. Each strain was cultivated and picked from BAK and suspended in prereduced demineralized water, by the methods described above. Each suspension was then diluted 10^3-fold in either demineralized water or saline. From the demineralized water suspension 2-10 µl samples were deposited (in triplicate) into MPRS, RTF, or VMG III (contained in virtually-filled 5 ml vials, without paper cones). The procedure required 10 minutes for each sample. After an additional 20 minutes (i.e., 30 minutes exposure to each diluent), 3-25 µl samples from the suspensions in demineralized water (undiluted and diluted samples), saline, MPRS, RTF and VMG III were deposited on BAK for enumeration (as previously described).

RESULTS

In Vitro Evaluation of MPRS, RTF and VMG III

Tables II:2 and II:3 represent the bacterial recoveries from either MPRS and RTF or from RTF and VMG III, at the designated storage times in those transport media in which vortex mixing was performed only once. Bacterial recoveries were evaluated by enumeration of total CFU on BAK, comparing the 0-hour value (which was expected to be about 30 CFU/deposit) with values at various storage times in that medium, and also comparing values between the two media simultaneously studied.

Some of the tested microorganisms could not be detected upon plating any of the 0-, 1-, 4- or 24-hour storage samples. These included *B. asaccharolyticus* (ATCC 25260 and ATCC 27067), *B. gingivalis* (ATCC 33277, W50, W83 and 6126), *B. melaninogenicus* (ATCC 25845), and *E. nodatum* (ATCC 33099). From these observations, it was unclear whether the cells failed to survive the transport media or the exposure to prereduced saline. (This question was addressed later--see below.) Most of the remaining microorganisms tested survived transport in either MPRS, RTF or VMG III, to variable degrees. *B. intermedius* (ATCC 25611), however, could not be recovered from MPRS, although it was recovered from RTF after being diluted in the same saline suspension. It also did not survive transport in VMG III (data not shown). *P. asaccharolyticus* (ATCC 29743) survived storage for one but not 4 hours in MPRS and RTF. In another trial it survived for 24 hours in VMG III but not in RTF. *P. saccharolyticus* (ATCC 14953) survived for 4 but not 24 hours in MPRS and RTF. *B. endodontalis* (BNIIA-F) survived for 4 but not 24 hours in RTF and VMG III, as did *B. oralis* (ATCC 33269), and *C. ochracea* (ATCC

27872) in one of two trials. Notably, the numbers of *S. anginosus* (SPIP 6IIA), *S. faecium* (ATCC 9790) and *S. sanguis* II (SPIP 5IIE) increased dramatically over 24 hours in VMG III as, to a lesser degree, did those of *F. nucleatum* (JP2, JN9 and JG16) in experimental comparisons between MPRS and RTF. Thus, behavior of strains was variable between the media and among the strains. For all other strains, data given in Tables II:2 and II:3 are representative of contrasts and trends observed in replicate experimental runs. For those bacteria which were relatively good survivors in these transport media, storage in RTF was generally better than in either MPRS or VMG III. These comparisons for the relatively good storage survivors are given in Tables II:2 and II:3.

Additionally, the data were expressed by histograms in which comparisons of the bacterial recoveries (CFU means) in each transport medium were made relative to initial CFU recoveries, defined as 100%, and were expressed as a function of storage time. CFU recoveries also were compared between transport media (Figs. 2-15) and statistically significant differences ($P<0.001$) were designated by an asterisk above the bars at analogous storage times. Furthermore, although the 0-hour values for both transport media were defined as 100%, a statistically significant difference between transport media could also occur at 0-hour storage times (e.g., Figs. 3, 5, 7-9, 11,12,14 and 15), because of differences in CFU means between any two transport media at identical storage times.

TABLE II:2. Statistical evaluation of bacterial recoveries (CFU means) and cell viabilities (% of initial) from MPRS and RTF.*

BACTERIA	STORAGE TIMES (HR)	CFU MEAN (± SEM)	% of Initial	# of Drops	STUDENT'S t Values**	
A. actinomycetemcomitans (ATCC 33384)						
MPRS	0	47.0 ± 2.5	(100)	11		
RTF		53.0 ± 1.1	(100)	12	2.34	NS
MPRS	1	10.0 ± 1.2	(21)	12		
RTF		22.0 ± 1.6	(41)	12	6.14	S
MPRS	4	5.4 ± 0.5	(11)	11		
RTF		22.0 ± 1.2	(41)	12	12.62	S
MPRS	24	0.0	(0)			
RTF		0.0	(0)			NS
B. fragilis (ATCC 25285)						
MPRS	0	120.3 ± 3.3	(100)	12		
RTF		100.3 ± 3.8	(100)	06	3.68	NS
MPRS	1	73.3 ± 2.3	(61)	12		
RTF		117.3 ± 7.7	(116)	04	7.29	S
MPRS	4	16.0 ± 0.8	(13)	12		
RTF		28.5 ± 1.9	(28)	06	7.01	S
MPRS	24	10.5 ± 0.9	(9)	12		
RTF		11.2 ± 0.9	(11)	12	0.58	NS

*Comparisons of Mean CFU/Deposit, % of Initial CFU and total # of CFU Deposits in MPRS and RTF.

** Statistically significant (S) where $p < 0.001$; nonsignificant (NS) where $p > 0.001$, in differences between CFU means. Statistical comparisons made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

(continued)

B. intermedius (ATCC 25611)						
MPRS	0	0.0		12		
RTF		24.6 ± 3.0	(100)	12		S
MPRS	1	0.0		12		
RTF		17.5 ± 1.7	(71)	12		S
MPRS	4	0.0		12		
RTF		7.5 ± 0.3	(30)	11		S
MPRS	24	0.0		06		
RTF		2.7 ± 1.3	(11)	06		NS
B. oralis (ATCC 33269)						
MPRS	0	29.3 ± 1.3	(100)	08		
RTF		52.6 ± 1.8	(100)	12	9.66	S
MPRS	1	39.3 ± 1.2	(134)	07		
RTF		62.5 ± 1.7	(119)	11	10.10	S
MPRS	4	8.0 ± 1.0	(28)	08		
RTF		18.1 ± 1.3	(34)	12	5.76	S
MPRS	24	0.6 ± 0.2	(2)	06		
RTF		9.9 ± 0.6	(11)	11	10.85	S
F.nucleatum (JN9)						
MPRS	0	16.7 ± 2.0	(100)	12		
RTF		17.9 ± 3.9	(100)	12	.74	NS
MPRS	1	22.3 ± 1.2	(134)	12		
RTF		22.2 ± 1.3	(124)	12	.03	NS
MPRS	4	36.2 ± 2.2	(217)	12		
RTF		27.0 ± 1.3	(151)	11	3.60	NS
MPRS	24	42.6 ± 2.6	(255)	12		
RTF		115.7 ± 3.9	(647)	12	16.31	S
P. asaccharolyticus (ATCC 29743)						
MPRS	0	22.2 ± 0.7	(100)	12		
RTF		30.7 ± 1.1	(100)	12	6.33	S
MPRS	1	7.9 ± 0.7	(36)	11		
RTF		19.1 ± 1.1	(62)	12	8.77	S
MPRS	4	0.0		12		
RTF		0.0		12		NS
MPRS	24	0.0		12		
RTF		0.0		12		NS

(continued)

P. saccharolyticus (ATCC 14953)						
MPRS	0	292.6 ± 11.4	(100)	10	4.72	S
RTF		226.3 ± 7.7	(100)	09		
MPRS	1	98.4 ± 4.2	(34)	09		
RTF		159.6 ± 6.1	(71)	11	7.27	S
MPRS	4	51.3 ± 2.2	(18)	10		
RTF		146.8 ± 3.7	(65)	12	20.85	S
MPRS	24	0.0		12		
RTF		0.0		12		NS
S. faecium (ATCC 9790)						
MPRS	0	55.0 ± 3.1	(100)	12		
RTF		62.1 ± 2.1	(100)	12	1.90	NS
MPRS	1	58.3 ± 1.9	(106)	12		
RTF		70.3 ± 3.1	(113)	12	3.32	NS
MPRS	4	55.1 ± 2.5	(100)	12		
RTF		68.5 ± 2.5	(110)	12	3.83	NS
MPRS	24	44.9 ± 2.7	(94)	12		
RTF		42.1 ± 1.8	(68)	12	0.67	NS
*S. faecium** (ATCC 9790)						
MPRS	0 (SEE ABOVE)					
RTF						
MPRS	1	69.6 ± 2.3	(127)	11		
RTF		85.1 ± 1.6	(137)	12	5.64	S
MPRS	4	81.8 ± 3.2	(149)	12		
RTF		97.5 ± 4.3	(157)	12	2.92	NS
MPRS	24	89.7 ± 2.4	(163)	12		
RTF		105.1 ± 3.7	(169)	12	2.98	NS

* 1 vial was used for all of the designated storage times in both MPRS and RTF.

TABLE II:3. Statistical evaluation of bacterial recoveries (CFU means) and cell viabilities (% of initial) from RTF and VMG III, and from MPRS and VMG III*.

BACTERIA	STORAGE TIMES (HR)	CFU MEAN (± SEM)	% of Initial	# of Drops	STUDENT'S t Values**	
A. actinomycetemcomitans (ATCC 33384)						
RTF	0	352.5 ± 56	(100)	04	10.35	S
VMG III		188.7 ± 22	(100)	12		
RTF	1	65.1 ± 3.5	(19)	12	9.19	S
VMG III		29.3 ± 1.8	(16)	12		
RTF	4	42.0 ± 2.6	(12)	12	8.46	S
VMG III		18.1 ± 1.1	(10)	12		
RTF	24	23.3 ± 1.9	(7)	12	3.58	NS
VMG III		15.2 ± 1.3	(8)	12		
A. naeslundii (ATCC 12104)						
RTF	O	12.3 ± 1.5	(100)	12	6.71	S
VMG III		2.1 ± 0.4	(100)	12		
RTF	1	2.8 ± 0.6	(23)	12	2.63	NS
VMG III		1.1 ± 0.4	(52)	12		
RTF	4	3.1 ± 0.8	(25)	12	2.07	NS
VMG III		1.4 ± 0.4	(67)	12		
RTF	24	1.2 ± 0.2	(10)	06	0.74	NS
VMG III		1.5 ± 0.4	(13)	06		
B. endodontalis (BNIIA-F)						
RTF	0	95.9 ± 6.1	(100)	12	9.63	S
VMG III		34.4 ± 1.8	(100)	12		
RTF	1	16.1 ± 1.6	(17)	12		
VMG III		24.4 ± 1.4	(71)	11	3.84	S
RTF	4	8.7 ± 0.9	(9)	06	10.37	S
VMG III		1.1 ± 0.3	(3)	12		
RTF	24	0.0		12		
VMG III		0.0		12		NS

*Comparisons of Mean CFU/Deposit, % of Initial CFU and total # of CFU Deposits in RTF and VMG III, and MPRS and VMG III.

**Statistically significant (S) where $p < 0.001$; nonsignificant (NS) where $p > 0.001$, in differences between CFU means.

Statistical comparisons made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

(continued)

B. fragilis (ATCC 25285)						
RTF	0	52.8 ± 1.8	(100)	12	12.99	S
VMG III		18.6 ± 1.8	(100)	12		
RTF	1	35.3 ± 3.4	(67)	12	1.69	NS
VMG III		28.1 ± 2.6	(151)	12		
RTF	4	51.0 ± 2.0	(98)	12	11.03	S
VMG III		20.3 ± 1.8	(109)	11		
RTF	24	29.2 ± 3.5	(56)	12		
VMG III		349.7 ± 70	(1880)	12		S
B. oralis (ATCC 33269)						
RTF	0	77.0 ± 8.5	(100)	09	1.96	NS
VMG III		57.3 ± 5.9	(100)	12		
RTF	1	60.3 ± 2.9	(78)	12	3.37	NS
VMG III		36.6 ± 6.4	(64)	12		
RTF	4	48.3 ± 2.0	(63)	12	1.18	NS
VMG III		43.4 ± 3.6	(76)	12		
RTF	24	65.7 ± 4.0	(85)	06	6.53	S
VMG III		30.1 ± 3.7	(53)	09		
B. oralis (ATCC 33269)						
RTF	0	36.2 ± 1.9	(100)	12	6.87	S
VMG III		19.3 ± 1.5	(100)	12		
RTF	1	31.7 ± 1.6	(88)	12	10.31	S
VMG III		10.6 ± 1.6	(55)	12		
RTF	4	26.3 ± 1.6	(73)	12	6.97	S
VMG III		10.5 ± 1.6	(55)	11		
RTF	24	0.0		12		NS
VMG III		0.0		12		
C. ochracea (ATCC 27872)						
MPRS	0	21.3 ± 1.7	(100)	12	7.30	S
VMG III		6.3 ± 0.8	(100)	10		
MPRS	1	5.3 ± 0.7	(25)	12	1.10	NS
VMG III		6.3 ± 0.6	(100)	11		
MPRS	4	1.7 ± 0.3	(8)	10	1.51	NS
VMG III		3.0 ± 0.2	(48)	06		
MPRS	24	0.0		12		NS
VMG III		0.0		12		

(continued)

C. ochracea (ATCC 27872)						
RTF	0	22.8 ± 1.2	(100)	12	10.84	S
VMG III		6.3 ± 0.8	(100)	10		
RTF	1	9.1 ± 0.9	(40)	11	2.32	NS
VMG III		6.3 ± 0.6	(100)	08		
RTF	4	2.2 ± 0.4	(10)	10	1.38	NS
VMG III		3.0 ± 0.2	(48)	06		
RTF	24	2.8 ± 0.4	(12)	11		NS
VMG III		0.0		12		
P. saccharolyticus (SPIP 1IIA)						
MPRS	0	17.1 ± 1.2	(100)	12	0.48	NS
VMG III		16.3 ± 1.1	(100)	12		
MPRS	1	17.3 ± 1.5	(100)	06	5.80	S
VMG III		6.3 ± 1.1	(39)	12		
MPRS	4	15.7 ± 0.9	(92)	12	0.74	NS
VMG III		17.2 ± 2.1	(106)	09		
MPRS	24	6.8 ± 1.0	(40)	11		
VMG III		11.8 ± 0.8	(74)	12	3.99	S
P. saccharolyticus (SPIP 1IIA)						
RTF	O	11.8 ± 0.5	(100)	12	5.77	S
VMG III		6.5 ± 0.8	(100)	12		
RTF	1	6.3 ± 0.8	(53)	12	2.89	NS
VMG III		3.7 ± 0.4	(57)	10		
RTF	4	4.5 ± 0.4	(38)	12		
VMG III		8.3 ± 1.6	(125)	06	3.26	NS
RTF	24	10.4 ± 1.5	(88)	05		NS
VMG III		0.0		06		
P. asaccharolyticus (ATCC 29743)						
RTF	O	13.7 ± 0.7	(100)	12		
VMG III		19.2 ± 1.3	(100)	12	2.99	NS
RTF	1	3.5 ± 0.6	(26)	08		
VMG III		16.5 ± 1.4	(86)	08	7.62	S
RTF	4	6.4 ± 0.9	(47)	07		
VMG III		7.8 ± 0.9	(41)	11	1.04	NS
RTF	24	0.0		12		
VMG III		3.4 ± 0.7	(18)	10		NS

(continued)

P. saccharolyticus (ATCC 14953)						
RTF	0	42.0 ± 1.8	(100)	07	14.99	S
VMG III		14.4 ± 0.9	(100)	11		
RTF	1	44.1 ± 2.7	(105)	11	11.08	S
VMG III		15.2 ± 1.2	(105)	12		
RTF	4	34.7 ± 2.6	(83)	06	6.27	S
VMG III		15.1 ± 1.8	(105)	07		
RTF	24	27.0 ± 1.5	(64)	08	5.50	S
VMG III		16.4 ± 1.2	(114)	10		
S. anginosus (SPIP 6IIA)						
MPRS	0	9.4 ± 0.9	(100)	12		
VMG III		11.1 ± 1.0	(100)	12	1.25	NS
MPRS	1	15.1 ± 1.3	(161)	12		
VMG III		16.4 ± 1.2	(148)	12	0.75	NS
MPRS	4	10.0 ± 1.0	(106)	08		
VMG III		21.7 ± 1.0	(195)	07	8.35	S
MPRS	24	7.1 ± 0.3	(76)	07		
VMG III		>5OO	(TNTC#)	08		S
S. anginosus (SPIP 6IIA)						
RTF	O	12.1 ± 1.0	(100)	12	0.69	NS
VMG III		11.1 ± 1.0	(100)	12		
RTF	1	6.5 ± 0.7	(54)	11		
VMG III		16.4 ± 1.2	(148)	12	6.80	S
RTF	4	7.9 ± 0.8	(65)	09		
VMG III		21.7 ± 1.0	(195)	07	11.18	S
RTF	24	5.2 ± 0.6	(43)	06		
VMG III		>500	(TNTC#)	08		S
S. faecium## (ATCC 9790)						
RTF	O	58.3 ± 5.6	(100)	06	7.11	S
VMG III		24.1 ± 2.1	(100)	12		
RTF	1	92.3 ± 3.4	(158)	06	62.44	S
VMG III		16.7 ± 0.7	(70)	12		
RTF	4	106.7 ± 4.6	(183)	06		
VMG III		121.3 ± 7.3	(503)	12	2.40	NS
RTF	24	116.5 ± 5.3	(200)	06		
VMG III		>1000	(TNTC)	12	S	

TNTC = Too numerous to count colony forming units.
##1 vial of RTF was used for 0, 1, 4 and 24 hr storage times.

(continued)

S. sanguis II (SPIP 5IIE)						
MPRS	O	1.8 ± 0.7	(100)	08		
VMG III		15.0 ± 1.4	(100)	12	6.47	S
MPRS	1	7.1 ± 0.6	(394)	12	6.01	S
VMG III		2.3 ± 0.3	(15)	09		
MPRS	4	2.5 ± 0.3	(138)	08		
VMG III		4.1 ± 0.8	(27)	07	2.15	NS
MPRS	24	2.1 ± 0.3	(100)	08		
VMG III		45.2 ± 3.0	(301)	12	12.64	S
S. sanguis II (SPIP 5IIE)						
RTF	O	1.2 ± 0.2	(100)	08		
VMG III		15.0 ± 1.4	(100)	12	6.96	S
RTF	1	7.1 ± 0.6	(394)	06	2.60	S
VMG III		2.3 ± 0.3	(15)	09		
RTF	4	2.6 ± 0.7	(216)	06		
VMG III		4.1 ± 0.8	(27)	07	2.00	NS
RTF	24	1.0 ± 0.4	(83)	06		
VMG III		45.2 ± 3.0	(301)	12	9.01	S

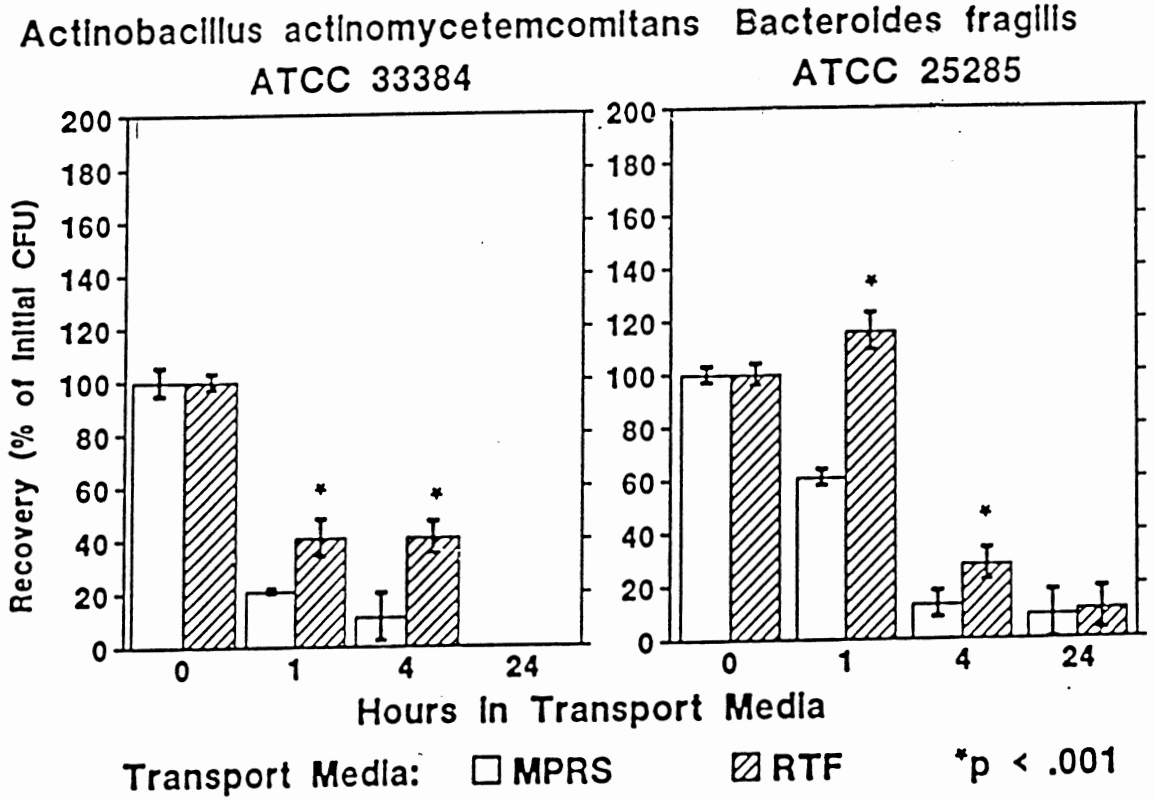

Figure 2. Percent of initial CFU recovery of *A. actinomycetemcomitans* (ATCC 33384) and *B. fragilis* (ATCC 25285) from MPRS and RTF, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

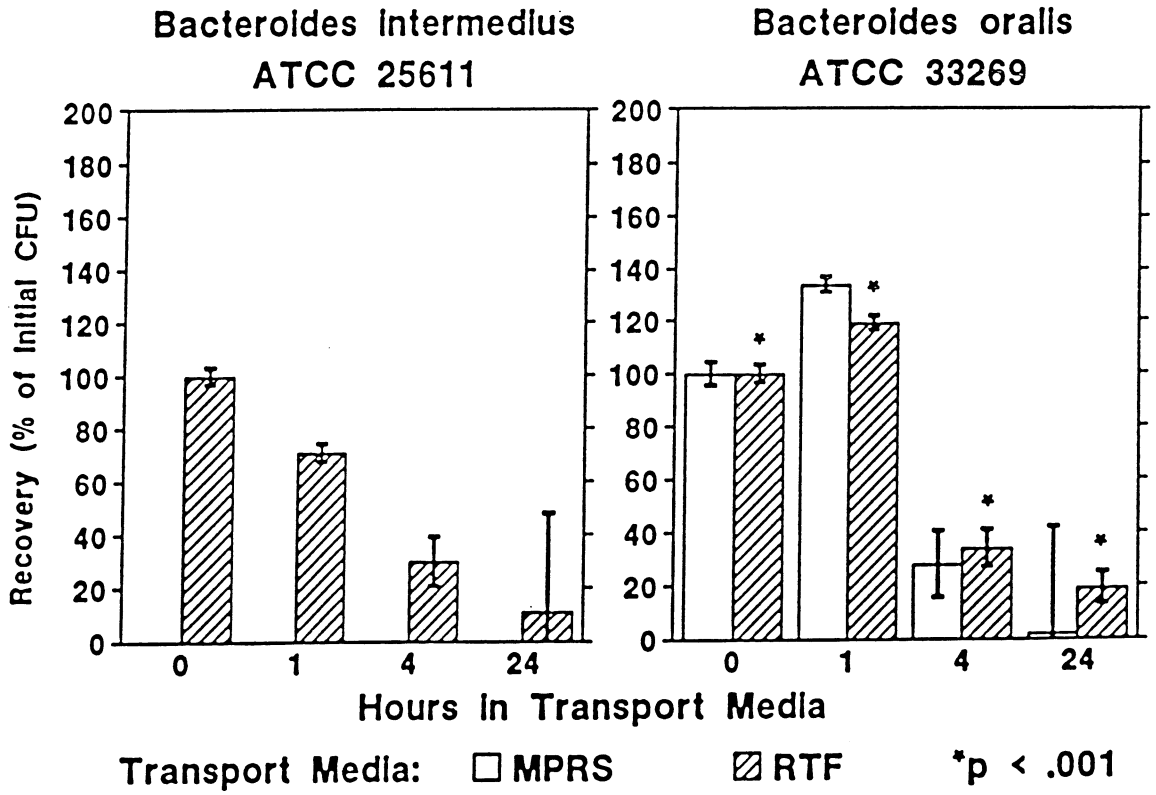

Figure 3. Percent of initial CFU recovery of *B. intermedius* (ATCC 25611) and *B. oralis* (ATCC 33269) from MPRS and RTF, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

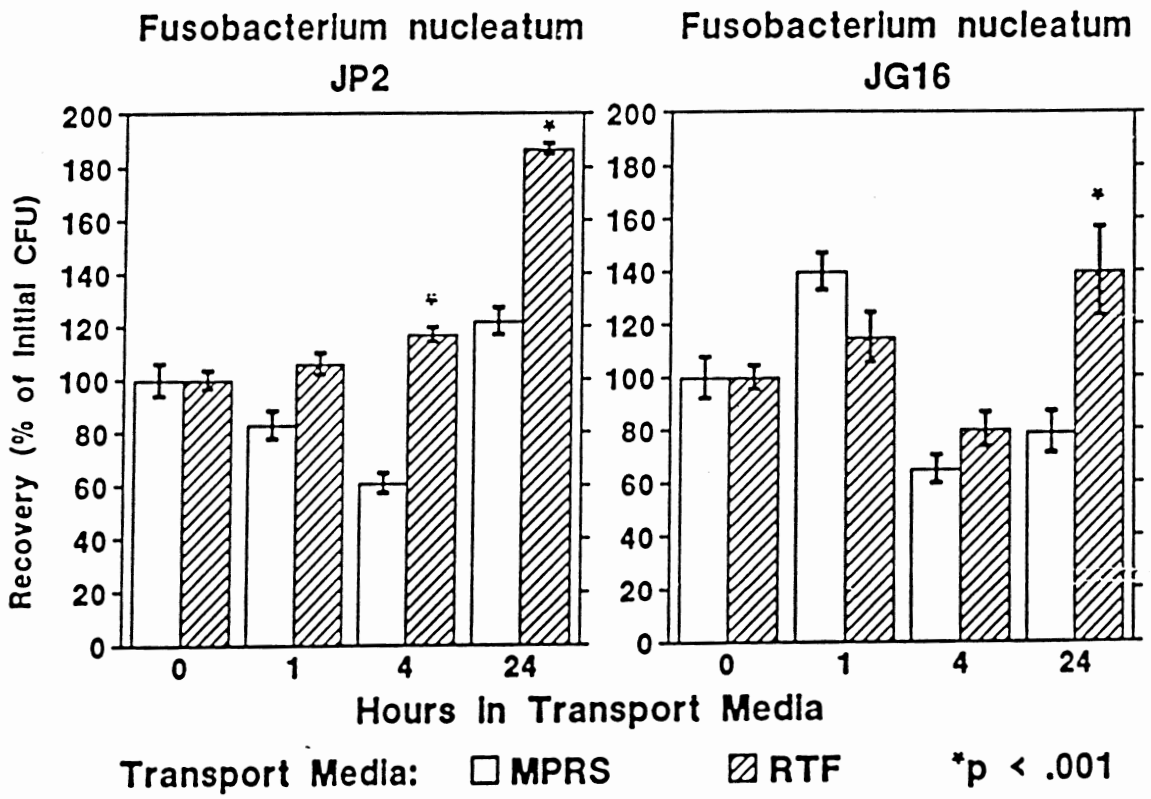

Figure 4. Percent of initial CFU recovery of *F. nucleatum* (JP2 and JG16) from MPRS and RTF, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

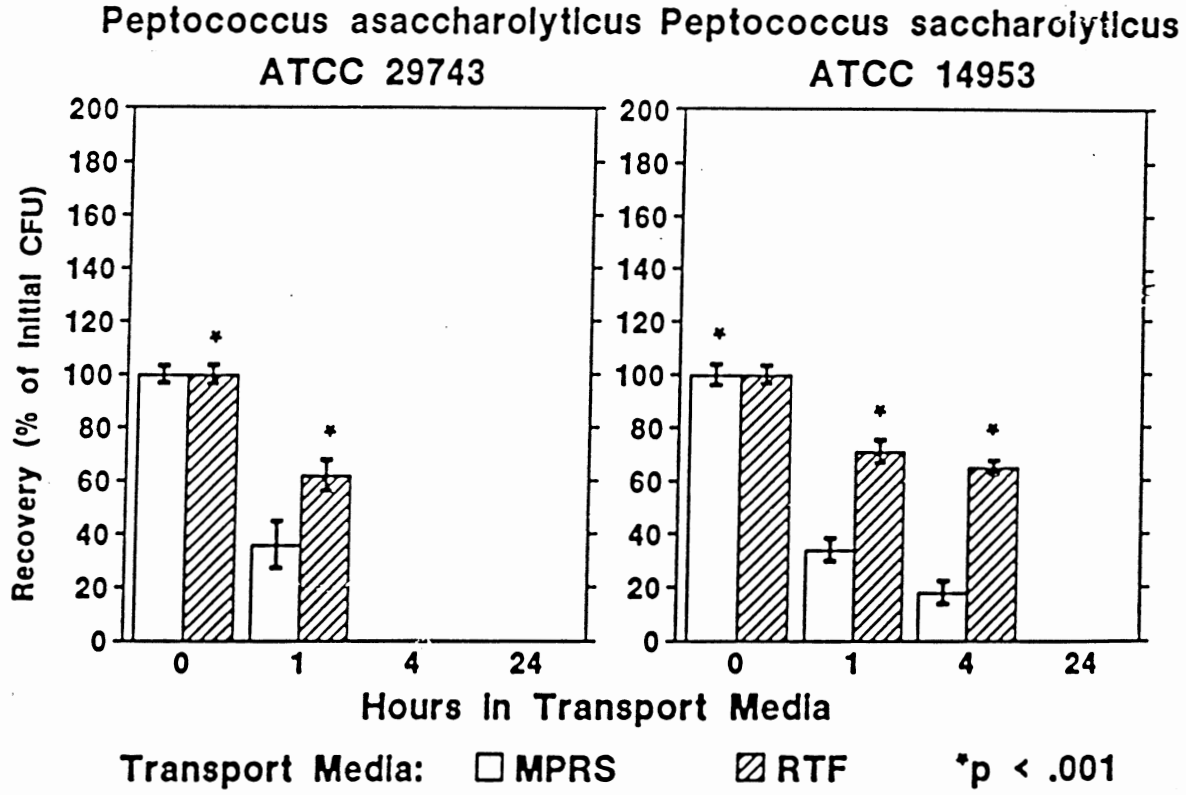

Figure 5. Percent of initial CFU recovery of *P. asaccharolyticus* (ATCC 29743) and *P. saccharolyticus* (ATCC 14953) from MPRS and RTF, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

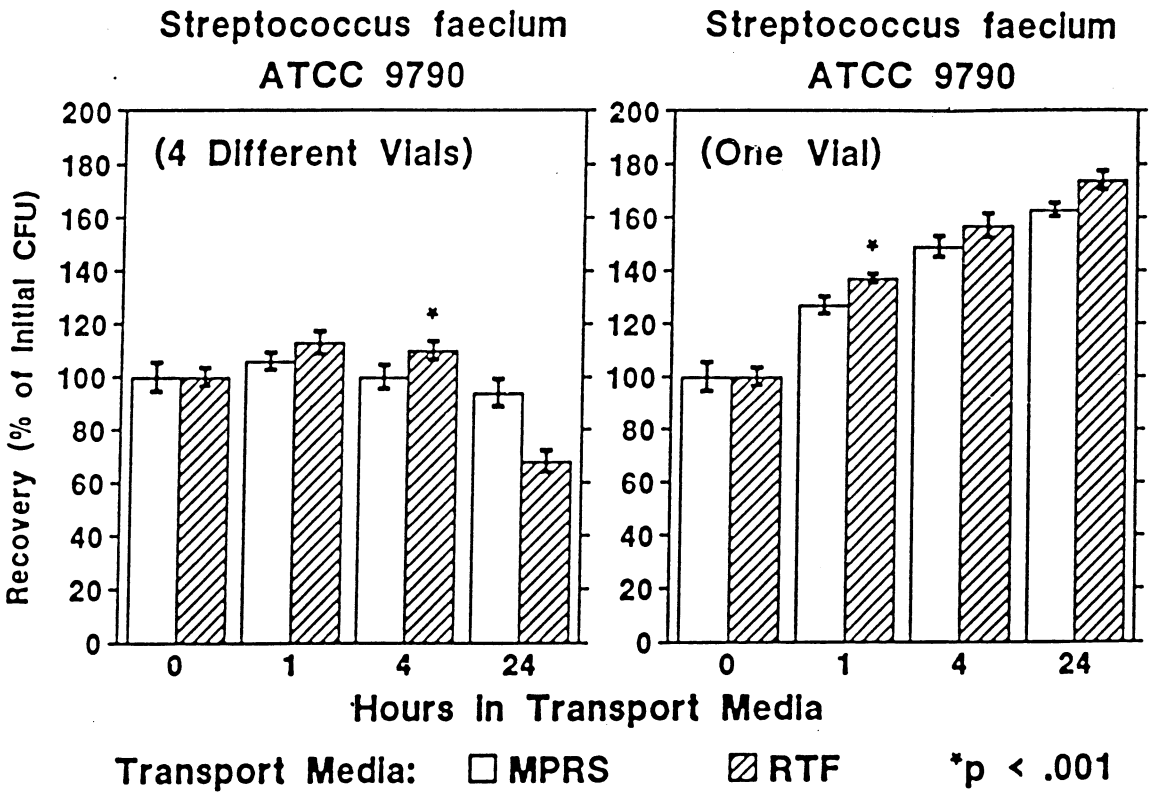

Figure 6. Percent of initial CFU recovery of *S. faecium* (ATCC 9790) from MPRS and RTF, in which 4 different vials were used for each storage time in the first evaluation, and 1 vial used 4 times for each storage time in the second evaluation.

This was done to discern the possible effect of repeated vortex mixing, in the presence of glass beads, on chain breakage.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

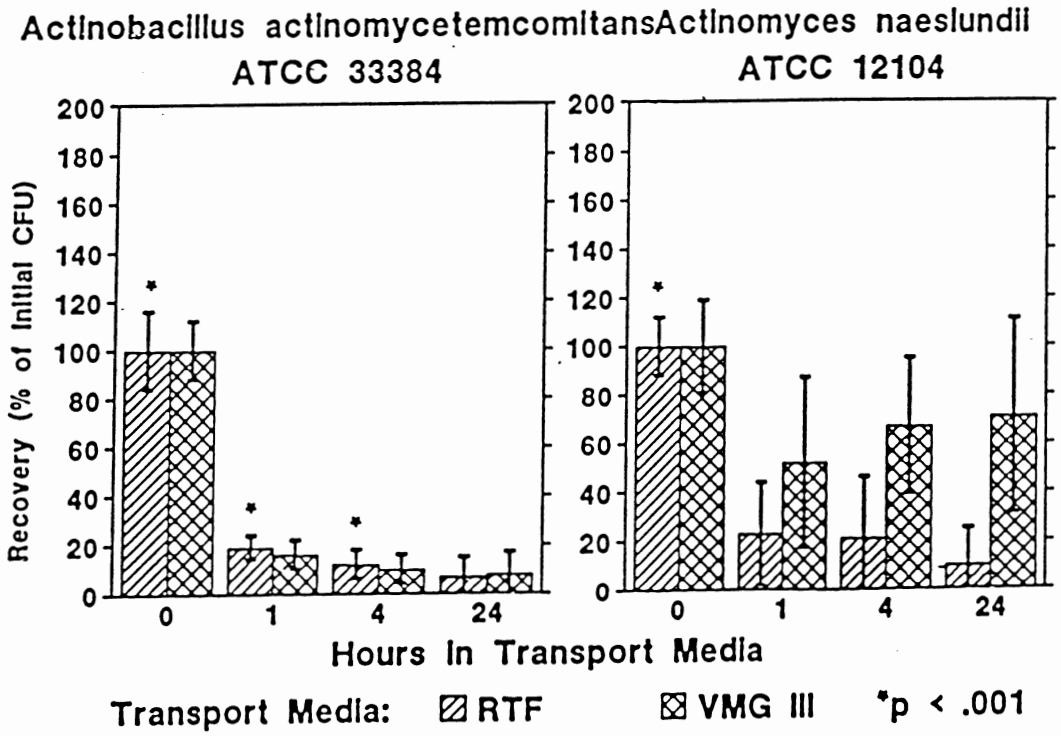

Figure 7. Percent of initial CFU recovery of *A. actinomycetemcomitans* (ATCC 33384) and *A. naeslundii* (ATCC 12104) from RTF and VMG III, as function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

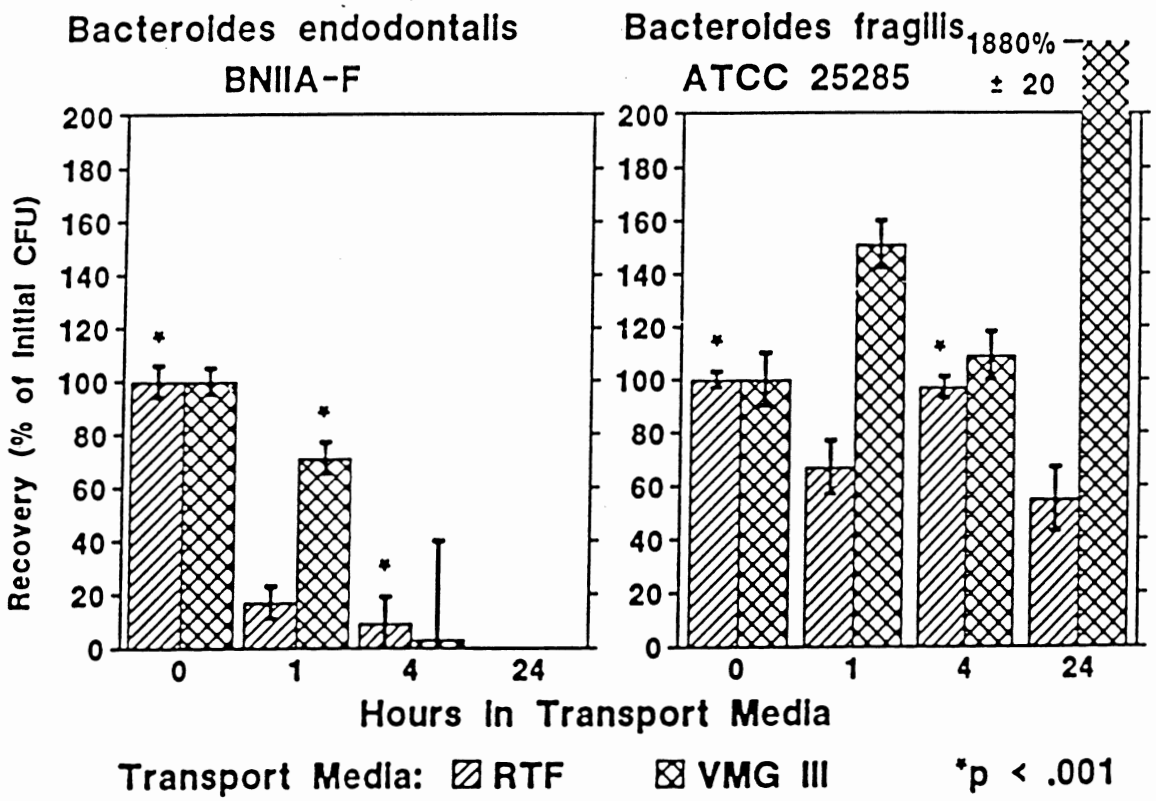

Figure 8. Percent of initial CFU recovery of *B. endodontalis* (BNIIA-F) and *B. fragilis* (ATC 25285) from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

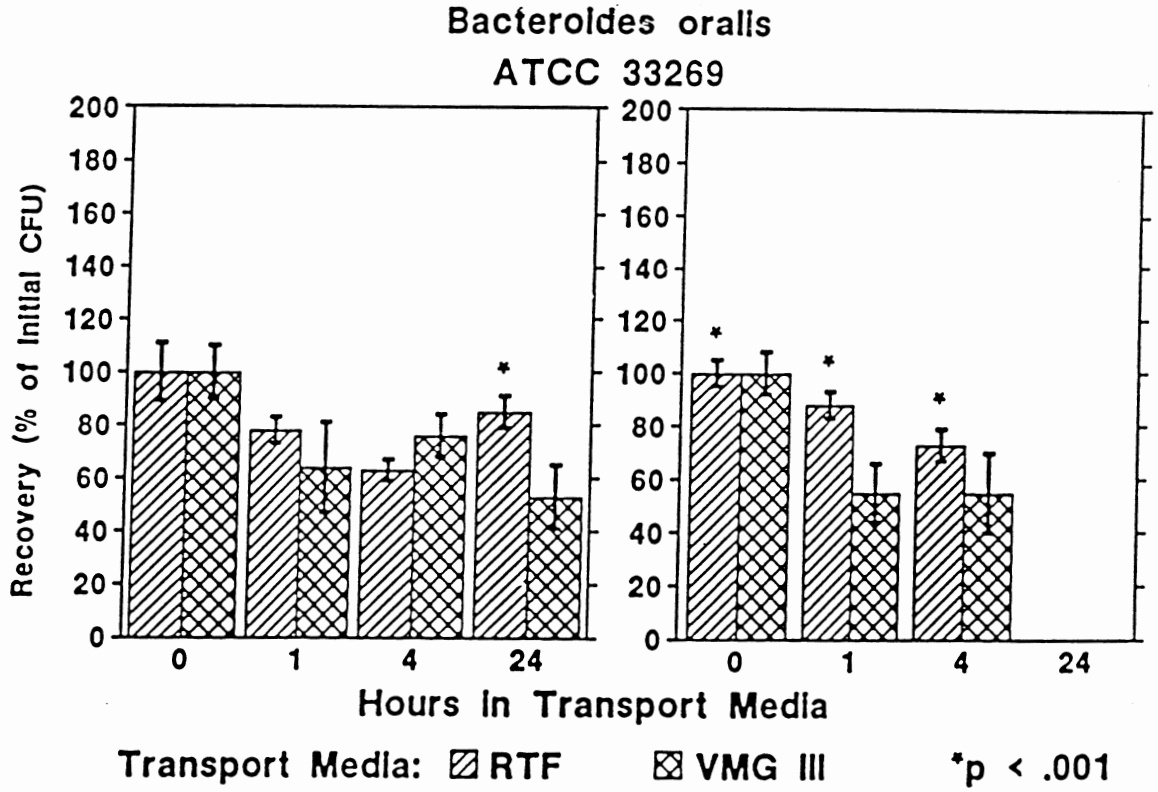

Figure 9. Percent of initial CFU recovery of *B. oralis* (ATCC 33269) from RTF and VMG III (in 2 independent trials), as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

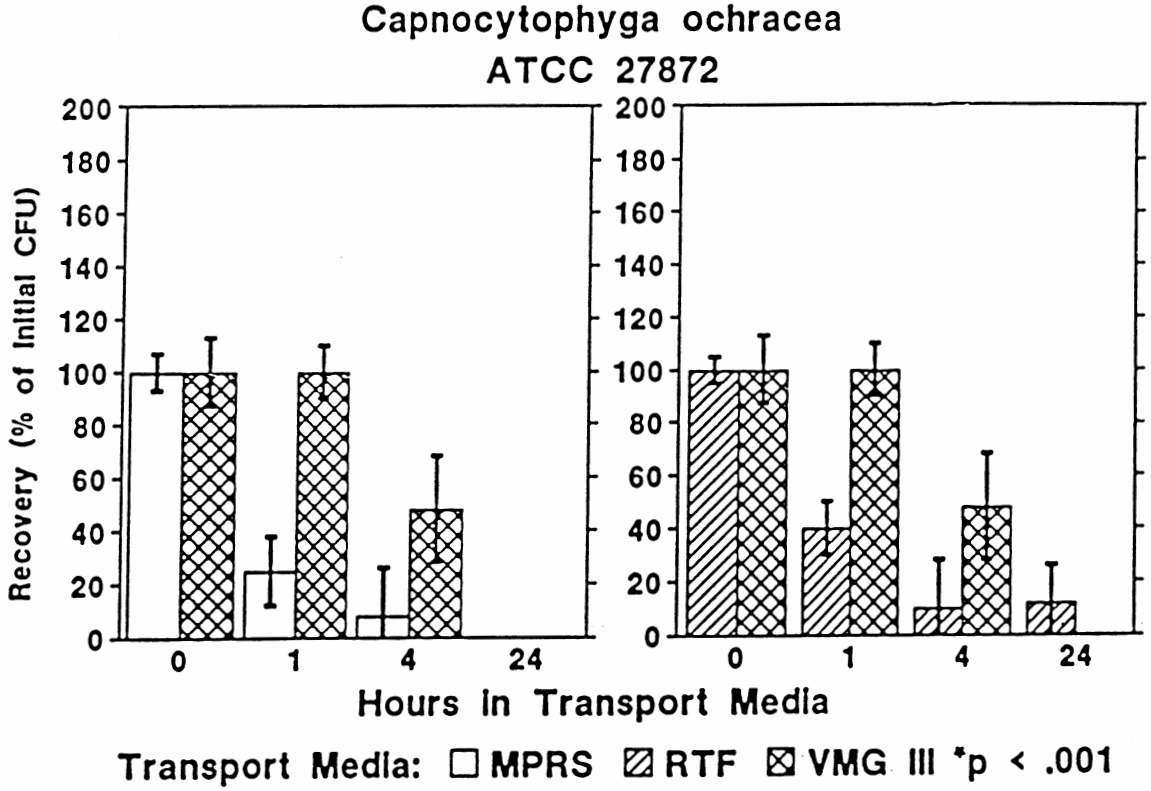

Figure 10. Percent of initial CFU recovery of *C. ochracea* (ATCC 27872) from MPRS and RTF, and from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

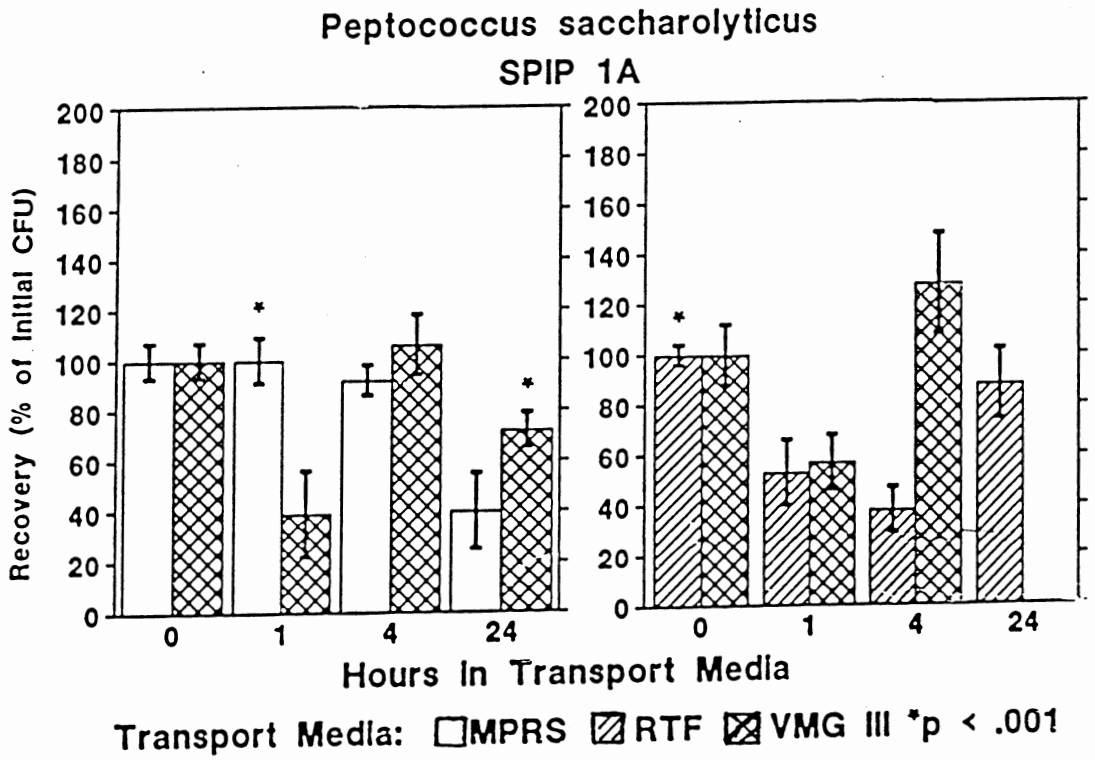

Figure 11. Percent of initial CFU recovery of *P. saccharolyticus* (SPIP 1IIA) from MPRS and VMG III, and from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

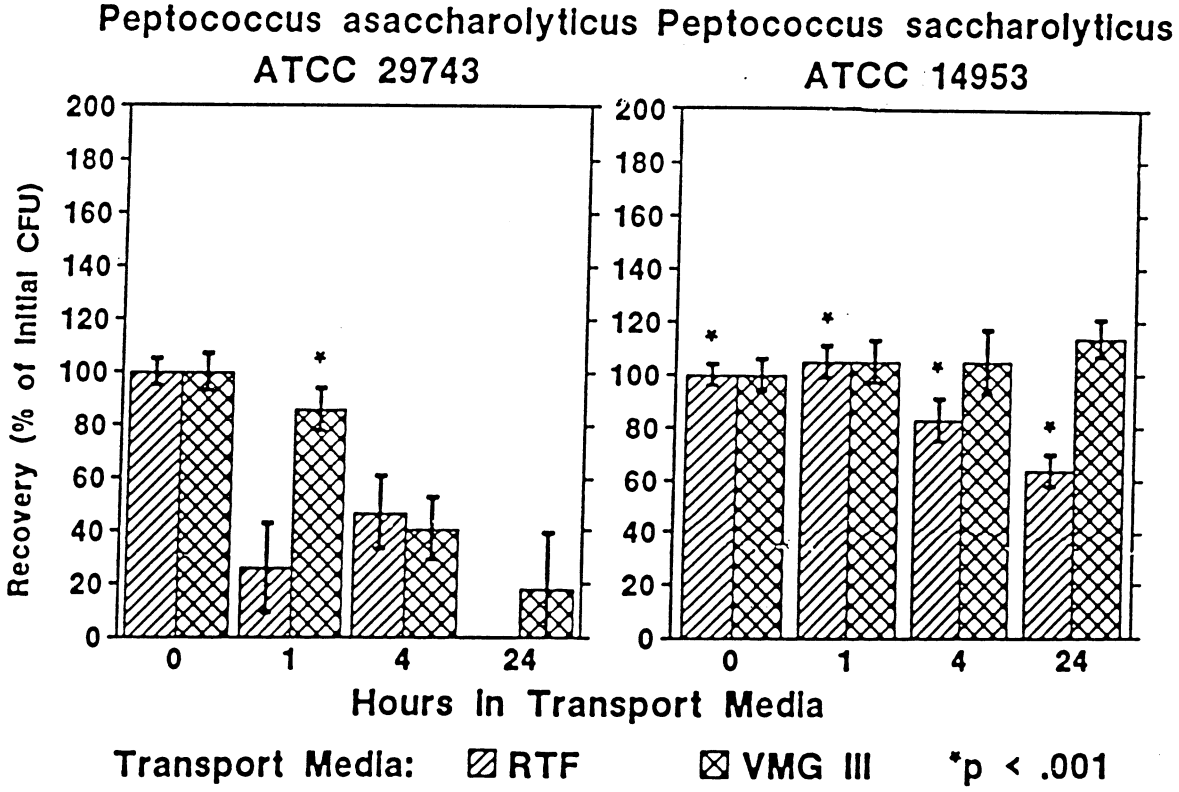

Figure 12. Percent of initial CFU recovery of *P. asaccharolyticus* (ATCC 29743) and *P. saccharolyticus* (ATCC 14953) from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

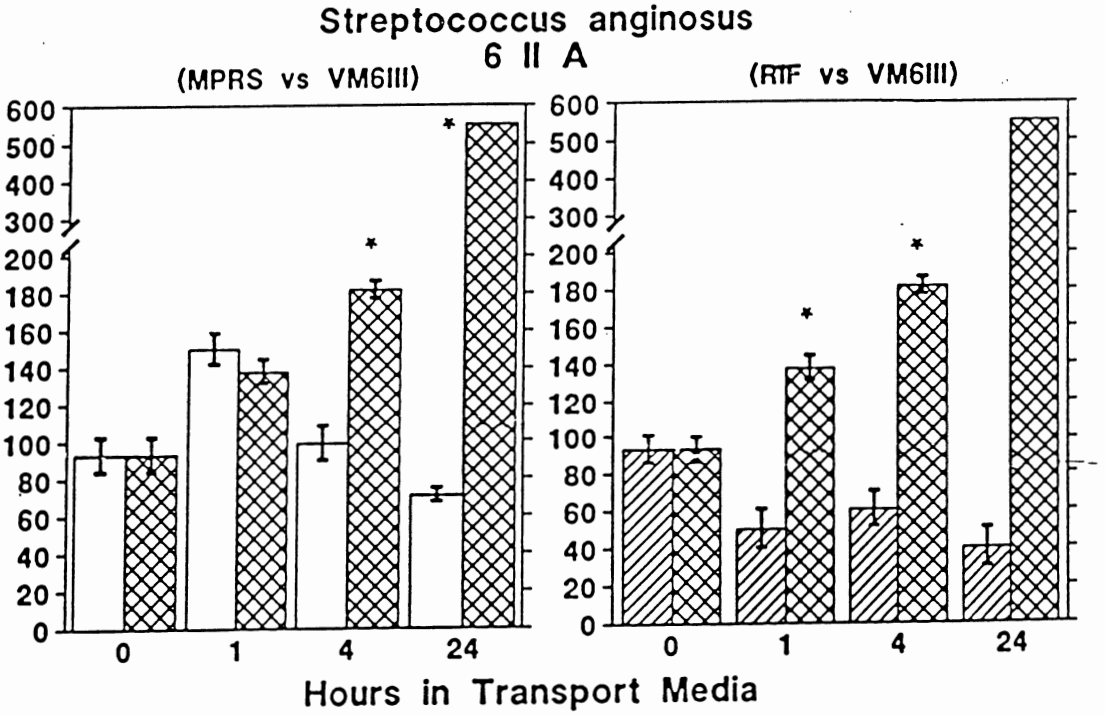

Figure 13. Percent of initial CFU recovery of *S. anginosus* (SPIP 6IIA) from MPRS and RTF, and from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

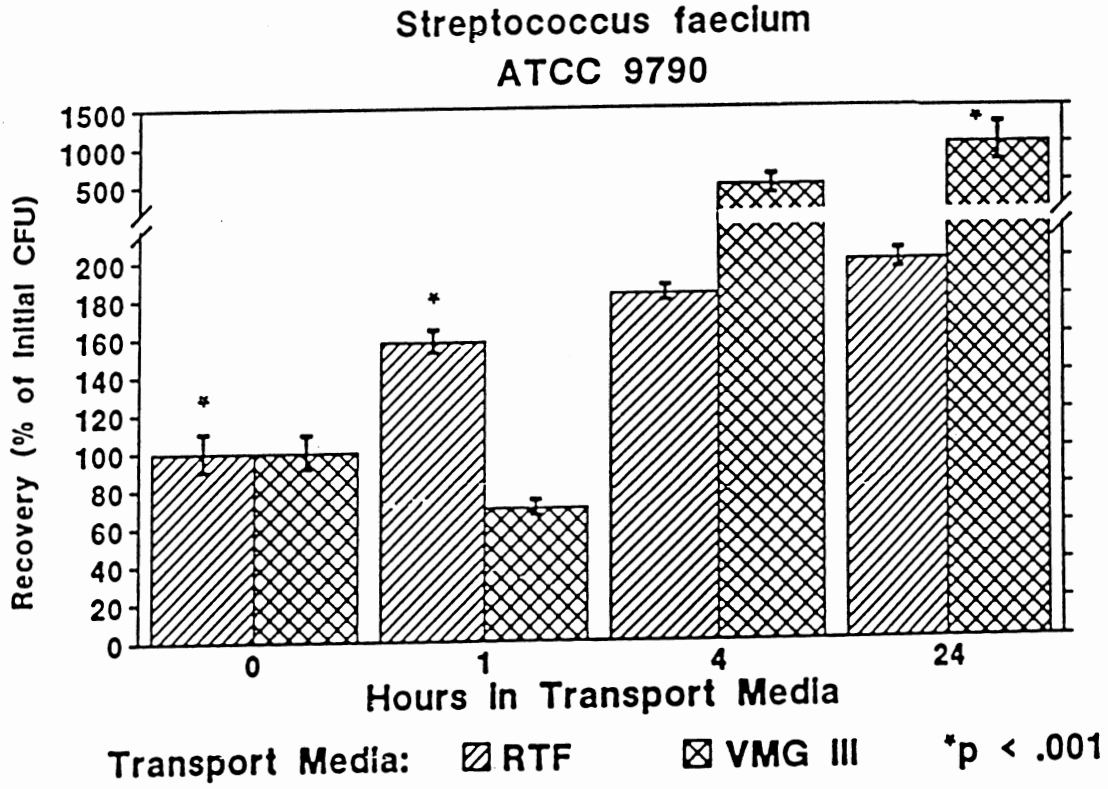

Figure 14. Percent of initial CFU recovery of *S. faecium* (ATCC 9790) in which 1 vial was used for RTF and 4 vials for VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium. Recall that repeated vortex mixing of a single vial (Fig. 6) fosters apparent chain breakage, as shown by increased CFU counts. A preliminary study had shown that increased CFU numbers in VMG III could not likely be accounted for by chain breakage alone and that growth in it could be occurring. Therefore the present trial was done with one vial of RTF, vortex mixed 4 times before sampling and 4 vials of VMG III, each vortex mixed only once. The dramatic increase in numbers of CFU in VMG III, is thus, likely to reflect growth in the medium.

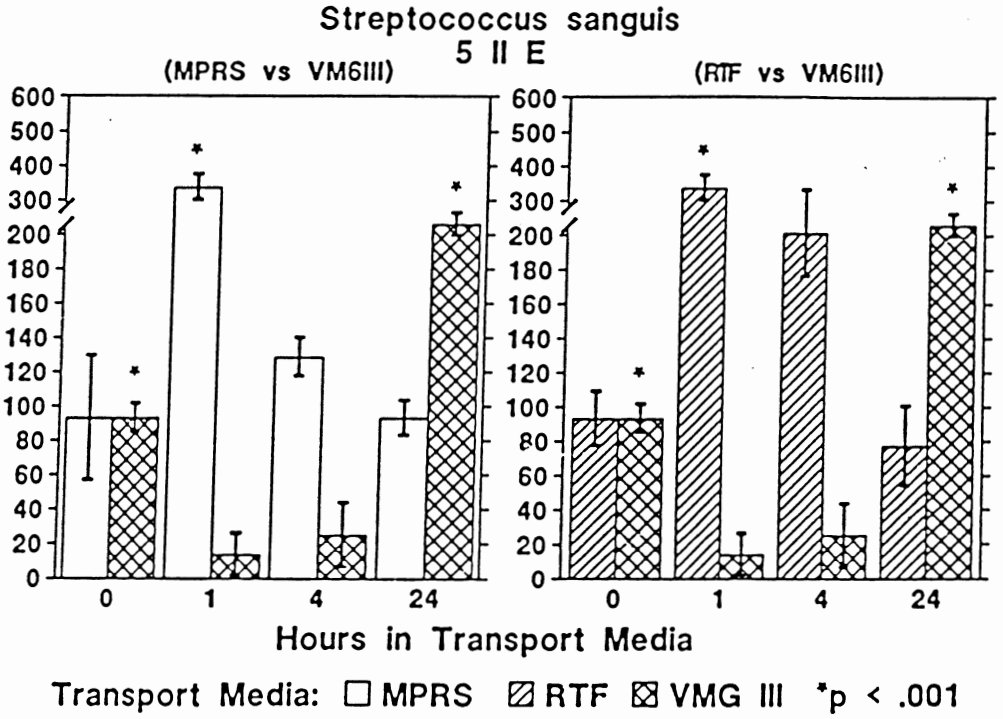

Figure 15. Percent of initial CFU recovery of *S. sanguis* II (SPIP 5IIE) from MPRS and RTF, and from RTF and VMG III, as a function of storage time.

Statistical comparisons were made only between the 2 transport media at identical storage times and not among the 4 storage times in each transport medium.

In Vitro **Evaluation of Effects of Exposure to Saline and Water**

To initially clarify the impact of suspension in prereduced saline on culture viability, triplicate samples had been deposited from saline suspensions directly onto BAK, after distribution of these suspensions to the transport media had been completed, as detailed above. The time lapse before deposition onto BAK was approximately 30 minutes. By this qualitative test of viability *A. naeslundii* (ATCC 12104), *B. gingivalis* (W50, W83, and 6126), *E. nodatum* (ATCC 33099), and *P. asaccharolyticus* (ATCC 29743) did not survive 30 minutes of saline exposure even though 25 µl deposits made on the agar should have contained about 7.5 x 10^3 cells and, thus, confluent growth should have occurred. In the case of *B. asaccharolyticus* (ATCC 25260 and ATCC 27067) nonconfluent growth was noted, suggesting severe loss of cells in the saline. Although 30 minute exposure to saline was apparently lethal to *A. naeslundii* (ATCC 12104) and *P. asaccharolyticus* (ATCC 29743), exposure for a lesser time was not lethal, as shown by survival upon subsequent transfer to transport media (see Tables II:2 and II:3). Because root canal samplings in our Endodontics Clinic are performed by flushing canals with (non-prereduced) isotonic saline, it was deemed important at this point to further scrutinize the effect of suspension of bacteria in saline or demineralized water in order to clarify whether loss of cell viability in saline could be ascribed to toxic salt effects (Ballantyne, 1930; DeMello *et al.*, 1951). The survival of 26 strains during 30 minutes of suspension in 5 diluents is presented in Table II:4.

Table II:4. Survival of 26 bacterial strains in a 30 minute suspension of pre-reduced demineralized water, saline, MPRS, RTF or VMG III.*

BACTERIA CFU/Deposits	H_2O (7.5×10^6)	H_2O	NaCl (7.5×10^3)	MPRS (3.0×10^1)	RTF	VMG III
A. actinomycetemcomitans (ATCC 33384)	++	++	++	##	##	##
A. naeslundii (ATCC 12104)	++	-	-	-	-	-
B. asaccharolyticus (ATCC 25260)	++	-	+	-	-	-
B. asaccharolyticus (ATCC 27067)	++	-	+	-	-	-
B. endodontalis (BNIIA-F)	++	++	-	#	##	-
B. fragilis (ATCC 25285)	++	++	++	##	##	##
B. gingivalis (ATCC 33277)	++	++	++	-	##	##
B. gingivalis (W50)	++	-	-	-	-	-
B. gingivalis (W83)	++	-	-	-	-	-
B. gingivalis (6126)	++	++	-	-	-	-

* The 1st suspension in demineralized water contains approximately 3×10^8 cells/ml; the 2nd suspension in demineralized water or in saline contains approximately 3×10^5 cells/ml, and are compared with only each other ; and the third suspension in either MPRS, RTF or VMG III contains approximately 1.2×10^3 cells/ml, and are compared with only each other.

++ = confluent growth.

+ = discrete colonies.

- = no colonies observed.

= observed number of CFU approximates expected numbers.

= observed numbers of CFU is decidely less than expected numbers.

(continued)

BACTERIA CFU/Deposits	H_2O (7.5×10^6)	H_2O (7.5×10^3)	NaCl	MPRS (3.0×10^1)	RTF	VMG III
B. intermedius (ATCC 25611)	++	++	++	-	##	##
B. intermedius (5W2)	++	+	++	#	#	#
B. melaninogenicus (ATCC 25845)	++	++	++	#	##	##
B. oralis (ATCC 33269)	++	++	++	##	##	##
C. ochracea (ATCC 27872)	++	++	++	-	-	-
E. nodatum (ATCC 33099)	-	-	-	-	-	-
F. nucleatum (JP2, JN9 & JG16)	++	++	++	##	##	##
P. asaccharolyticus (ATCC 29743)	++	+	-	-	-	-
P. saccharolyticus (ATCC 14953)	++	++	++	#	#	#
P. saccharolyticus (SPIP 1IIA)	++	++	++	##	##	##
P. anaerobius (ATCC 27337)	++	++	++	#	##	#
S. anginosus (SPIP 6IIA)	++	++	++	##	##	##
S. faecium (ATCC 9790)	++	++	++	##	##	##
S. sanguis II (SPIP 5IIE)	++	++	++	##	##	##

++ = confluent growth.
+ = discrete colonies
- = no colonies observed.
= observed number of CFU approximates expected numbers.
= observed numbers of CFU is decidely less than expected numbers.

Most strains survived in 10 ml suspensions of demineralized water (undiluted and diluted cell suspensions) and saline (diluted from the undiluted demineralized water suspensions), but some strains failed to survive when subsequently diluted in 5 ml of either MPRS, RTF or VMG III. By contrast, *B. intermedius* (5W2) and *B. asaccharolyticus* (ATCC 25260 and 27067) were better recovered from saline than from water, although recoveries were lower than expected for the two *B. asaccharolyticus* strains. Contrasts between observed and expected survivals in the three transport media for 30 minutes, after previous 10 minute exposure to water, are also given in Table II:4. Most notable are the failures of three *B. gingivalis* strains to survive in all the media and the virtual failure of the two *B. intermedius* strains to survive in MPRS. One strain of *C. ochracea* (ATCC 27872) also failed to survive in all the transport media, as did one of *P. asaccharolyticus* (ATCC 29743). However, this last failure could be attributed to failure to survive in water.

***In Vivo* Evaluation of 4 Teeth with Necrotic Pulps**

To evaluate the predictions made by the *in vitro*-studies, that RTF is likely to be a better transport medium than MPRS for those microorganisms that can be recovered from both, MPRS and RTF were used to transport samples from intact teeth with necrotic pulps before root canal instrumentation. The culture procedures used were as previously described, except that two paper cones were deposited into vials containing either 5 ml of MPRS or RTF after placement into the newly opened pulp chambers, and the order of placement of the cones into the transport fluids was alternated to minimize the effect of bias due to sequence of sampling. In each case, the same bacterial species were recovered from both transport media; therefore, the results given in Tables II:5

and II:6 are representative of those for either medium. Eight bacterial strains were recovered in 3 of 4 necrotic pulps with 2 of 8 being facultative anaerobes (*S. anginosus* and *A. viscosus)* and the remaining being strict anaerobes (*Streptococcus* sp., *Bacteroides*-like sp., *Lactobacillus*-like spp., and *F. nucleatum*). No spirochetes or motile forms were detected in stained or unstained smears of these 4 teeth.

Table II:5 summarizes the clinical findings associated with the isolated microflora of the 4 teeth cultured.

TABLE II:5. Summary of clinical and bacteriological findings in 4 teeth with necrotic pulps*.

SUBJECTS	GENDER /AGE	TOOTH ID#	PAR/ SIZE	WL FILE BEYOND APEX**	MICROFLORA ISOLATED
NP #1	M/37	4.1	YES/4	YES	*S. anginosus* *Streptococcus* sp. (anaerobic)
NP #2	F/36	4.1	YES /10	YES	*Streptococcus* sp. (anaerobic) *Bacteroides* sp.
NP #3	M/23	1.1	YES/7	NO	*A. viscosus* *F. nucleatum* *L. fructivorans* *Lactobacillus* sp.
NP #4	M/19	1.1	NO	NO	NONE

* NP= intact teeth with necrotic pulps.
** Tooth identification according to the Federation Dentaire International designation. PAR= periapical radiolucency/size in mm (diameter). WL= working length of root canal file placed beyond the apex (by radiographic criteria).

Table II:6 summarizes the key bacteriological findings in each tooth cultured. For the interested reader, the individualized data for each patient and the evidence leading to tentative identification of bacteria isolated from root canal cultures are given in Appendix C, Tables C:12 through C:15.

TABLE II:6. Key characteristics for identification of NP isolates*.

Organism	Gram Stain	Hem. on BAK	Ob/Fac Anaerobe	Fermen-tation	Biochem Rxns
S. anginosus (NP 1IIA)	+	-	Fac	2,3,4,7, 9,13,15,16	b,e
Streptococcus sp. (NP 1IIB)	+	-	Ob	NONE	b
Streptococcus sp. (NP 2IIA)	+	-	Ob	3,6,7,9, 13,15	NONE
Bacteroides sp. (NP 2IIB)	-	B	Ob	3,9	a,d
A. viscosus (NP 3IIA)	+	-	Fac	3,4,6,7, 9,11,15	a,b,g
Lactobacillus spp. (NP 3IIB and 3IIC)	+	-	Ob	3	NONE
F. nucleatum (NP 3IID)	-	-	Ob	3,4	d

* For Gram stain characteristics + = a positive reaction, - = a negative reaction; for hemolysis characteristics A = alpha-hemolysis, B = beta-hemolysis, and - = no detectable hemolysis; for anaerobic characteristics Ob = obligate anaerobe and Fac = facultative anaerobe.The following numbers correspond with positive fermentation of carbohydrates: 1= arabinose, 2= cellobiose, 3= fructose, 4= glucose, 5= inositol, 6= lactose, 7= maltose, 8= mannitol, 9=mannose, 10= melibiose, 11= raffinose, 12= rhamnose, 13= salicin, 14= sorbitol, 15= sucrose, 16= trehalose.The following letters correspond with positive biochemical reactions: a= catalase activity, b= esculin hydrolysis, c= gelatin liquefaction, d= indole production, e= litmus milk curd production, f= litmus milk hydrolysis, g= nitrate reduction, h= starch hydrolysis and i= urease activity.

DISCUSSION

The PCT method used for evaluating the efficacy of bacterial transport in 3 media could have produced considerable variability in the data. However, the following two procedures were used to compensate for procedural bias: Each bacterial suspension (in each transport medium) was diluted to provide approximately 30 CFU/deposit onto BAK agar, and all strains were studied at least twice, on different days. This was done not only to test the viability-preserving potential of transport media at conditions analogous to those seen clinically, that is, when few bacteria are present in a sample (Study I), but also, so as to avoid counting too many or too few CFU/deposit, shown to increase variance of CFU estimations (Westergren and Krasse, 1981). In general, there was good duplication of the trends of each run, although absolute values as a function of time did vary. Only one of 2 or 3 runs was shown in tables or graphs; however, both sets of data (in Fig. 9 and Table II:3) are given for *B. oralis* (ATCC 33269) to demonstrate the variability observed in a typical set of runs. In addition, so that comparisons of the efficacy of transport media could be interpreted with maximal caution, the highly conservative significance level of $p < 0.001$ was adopted in tests between media studied simultaneously.

It is uncertain why CFU recoveries were generally higher in RTF than MPRS because both are salt solutions (Study I, Table II:3). MPRS, however, contains 1% sodium metaphosphate, an agent reported to be significantly antibacterial (Tanzer and Hageage, 1970), and the concentration of sodium chloride in it is nearly twice that in RTF. According to Möller (1966) sodium ions are toxic to certain bacteria. Nonetheless, there are other differences between

these two media which could also affect cell viability. Thus, to address the question of the precise source of toxicity, further study would be required.

Because MPRS was generally less efficacious than RTF, VMG III was then compared with RTF. The methods previously used for comparisons between MPRS and RTF were slightly modified because VMG III contains agar and gelatin which cause it to solidify at room temperature. Upon warming, the medium becomes semisolid and the contents could then be vortex mixed and distributed. RTF generally demonstrated higher CFU recoveries than VMG III at each storage time. It is not clear why. Possible explanations include inhibition of release of cells from paper cones, inhibition of bacterial distribution, and/or adherence to the pipette due to the medium's viscosity. All of these possibilities would foster increased variance in estimates of the bacterial contents in samples taken from it. Higher variance of samples taken from VMG III than RTF, however, were not demonstrated. In all 3 transport media, the numbers of *S. faecium* (ATCC 9790) increased. This was shown to be a function of the number of times a sample was vortex mixed (with glass beads). Because of these observations, studies were done with four replicate vials, each vortex mixed just once. Despite this, there were dramatic increases in CFU recoveries, during 24-hour storage times, of *S. anginosus* (SPIP 6IIA), *S. faecium* (ATCC 9790), and *S. sanguis II* (SPIP 5IIE) in VMG III, which likely reflected growth of the streptococci in this medium. Moreover, *B. fragilis* (ATCC 25285) also grew in VMG III but not in RTF or MPRS. This behavior may be due to the proteinaceous substances contained in VMG III (Gästrin *et al.*, 1968).

Although reproducibility of data was good within a strain, it was often poor among strains of the same species. For example, CFU recoveries of *B. gingivalis* (ATCC 33277) were completely different from those of other strains of *B. gingivalis* (W50, W83 and 6126). Similarly, *B. intermedius* (ATCC 25611) had different recoveries than did another strain of *B. intermedius* (5W2). Furthermore, it was surprising that for several types of bacteria few CFU or, indeed, no CFU were recovered in 25 µl deposits on BAK plates. This suggested that the observed significant loss of cells could have occurred during suspension in prereduced saline and, therefore, cell loss could not be unambiguously ascribed to failure of transport media in these cases. These observations led to studies comparing suspension of BAK-grown colonies in demineralized water and, subsequently, resuspension in saline, water, MPRS, RTF, or VMG III, for enumeration after 30 minutes exposure in these fluids. *E. nodatum* (ATCC 33099) survived none of these procedures, and several strains either were completely lost or unexpectedly diminished in numbers in water and/or saline. This raises several questions about the use of saline or water as sampling fluids in root canal cultures. Some investigators used growth media (chopped meat broth with or without glucose) for sampling root canal contents (Berg and Nord, 1973; Bergenholtz, 1974; Griffee *et al.*, 1980). Although this could conceivably be of benefit in retrieving fastidious bacteria from canals (this has never been reported), it would seem to be hazardous for its risk of inducing allergic sensitization of patients who are subject to repeated exposure to root canal therapy and, consequently, canal contents sampling. These findings recommend that sampling of canals should optimally avoid use of all of these sampling fluids. Sterile files or paper cones (instrumented with

sterile-gloved fingers or hemostats) could be dropped directly into prereduced transport medium-containing vials which are immediately sealed, or paper cones could be rolled onto agar immediately after removal from the root canal. It is recognized, though, that the absence of fluid during bacterial sampling may result in oversight of microorganisms in dentinal tubules, and that direct inoculation onto agar with paper cones was reportedly less efficacious than the use of transport media (Möller, 1966). Nonetheless, the compromise of using a fluid-less sampling procedure may be a lesser risk. In view of these problems of saline and water use for canal sampling it should not be surprising that in Study I, using MPRS as a sampling fluid, limited numbers of microorganisms were detected. It is doubtful, however, that this sampling effect could have accounted for the nonrecovery of microorganisms from 5 of the 10 SPIP cultures because no evidence of microorganisms was detected by direct microscopy. These observations led to a clinical comparison between MPRS and RTF by culture of newly-opened necrotic pulps sampled with paper cones, after use of the PMR procedures in the presence of MPRS. There were no detectable differences in recovery results using the two media. In addition, 6 of 8 isolates recovered from both media were strict anaerobes. Subsequent sampling of canals with saline-paper cones and transport in thioglycollate medium, as are standard clinical procedures, however, yielded recovery of only some of the anaerobic bacteria found in MPRS and RTF. This cannot be unambiguously attributed to sequence of the canal sampling, failure of the saline sampling fluid, or failure of the thioglycollate broth. This observation is consistent with that in Study I in which

MPRS but not saline-thioglycollate recovered anaerobes. It suggests that sampling of canals in routine endodontic practice should at least abandon the saline-thioglycollate sequence of procedures.

One of the NP patients (#2) was subsequently seen to develop SPIP (SPIP #10). It is perhaps of interest that on sampling of the necrotic pulp (i.e., at initial instrumentation) an anaerobic *Streptococcus* and a *Bacteroides*-like species were the only bacteria recovered. However, after occurrence of SPIP, no bacteria were recovered or observed in the root canal.

This suggests a bacteriological predictor of SPIP but does not confirm it. Indeed, so few organisms may be present after initial instrumentation as to escape detection (or be lost by the procedures) upon reculture of the tooth.

It is of further interest that 2-4 types of bacteria were isolated from 3 of 4 NP canals and that among them was a beta-hemolytic *S. anginosus* together with an anaerobic streptococcus in one canal, an anaerobic streptococcus and a pigmented bacteroid in another, and *A. viscosus, F. nucleatum, L. fructivorans* and a seemingly anaerobic *Lactobacillus* from the third. Most of these bacteria have previously been reported isolated from necrotic pulps (Sundqvist, 1976; Yoshida *et al.*, 1987). Because VMG III was not used for clinical samples of the 4 NP teeth, and because *in vitro* evaluation of this medium was performed with 5 ml vials (containing 1 mm glass beads) rather than the smaller vials (containing 3 mm glass beads) used by Möller (1966) and others, there is a small measure of uncertainty about the efficacy of this medium. Nonetheless, this medium did not perform as well as RTF *in vitro* for bacterial cell recoveries.

It is difficult to account for loss of detectable viability of cultures based upon dilution effects only. However, the data suggest that viability loss is disproportionately great when inocula are small, despite initial numbers of cells which should have been more than adequate for ready detection of colonies upon inoculation of plates.

SUMMARY

The transport medium used in Study I, MPRS, was compared with another salt-solution medium, RTF, and this, in turn, was compared with VMG III, to assess their comparative efficacy for bacterial transport. Selection of bacteria for study was based upon reported isolation of some from root canals and their fastidious nutritional requirements. An *in vitro* model (PCT) was developed with the intent of simulating culture procedures performed clinically. Storage times of 0, 1, 4 and 24 hours were arbitrarily selected for evaluation of bacterial recoveries. Comparisons of trends from initial CFU recoveries within each transport medium and among transport media were evaluated during the selected storage times. The recoveries of various bacterial strains stored for up to 24 hours in either MPRS, RTF or VMG III varied considerably within the same strains and among different species. RTF, however, generally demonstrated higher CFU recoveries than the other two media. Some strains failed to survive when subjected to a 30 minute exposure in either saline or water. Three of 4 subjects with necrotic pulps had recoverable bacteria at initial root canal samplings. Two to 4 bacterial types were recovered from each of 3 positive canals. There was no detectable difference in results when MPRS and RTF were used as transport media; however, saline-thioglycollate transport was less successful in recoveries of microorganisms.

CONCLUSIONS

This series of experiments evaluated bacterial recoveries and cell viabilities in MPRS and RTF, and then in RTF and VMG III, for up to 24 hours storage. The following was concluded:

1. CFU recoveries were statistically ($p<0.001$) greater in RTF than in either MPRS or VMG III for most of the strains that survived storage times of 0, 1, 4, and 24 hours. For some bacteria, at storage times greater than 4 hours, VMG III clearly supports proliferation, thus acting as a growth medium rather than a transport medium. Its use could, therefore, significantly distort the results of root canal cultures.
2. The following strains did not survive 30 minute suspension in prereduced saline, MPRS, RTF or VMG III: *A. naeslundii* (ATCC 12104), *B. gingivalis* (W50, W83 and 6126), *E. nodatum* (ATCC 33099), and *P. asaccharolyticus* (ATCC 29743). All strains except *E. nodatum* (ATCC 33099) survived 30 minute suspension in prereduced demineralized water.
3. It would appear reasonable that one directly deposit the last root canal file, handled with strict aseptic technique, into sealed vials of RTF. Saline, water, or MPRS as sampling fluids appear highly problematic, especially after initial root canal instrumentation. Although not directly tested in this study it would appear unwise to inject any antigenic-containing substances into a root canal for use as a sampling fluid, because of the possible risk of allergic sensitization.

4. Cultures of 4 teeth with necrotic pulps, using either MPRS or RTF as transport media, were qualitatively similar. The following bacteria were recovered from both: *A. viscosus, Bacteroides*-like species, *F. nucleatum*, an unidentified *Lactobacillus*-like species, *L. fructivorans, S. anginosus*, and two strains of anaerobic streptococci.
5. No spirochetes or motile forms were detected in stained smears or wetmounts of these 4 samples.
6. Subsequent culture of these 4 teeth with thioglycollate broth failed to recover the *Bacteroides*-like species or anaerobic streptococcal species recovered from the transport media. Combined with the findings of Study I, it appears that clinical endodontics should abandon saline-thioglycollate sampling of canals.
7. It would appear important to develop prospective data on the predictors of SPIP.

APPENDICES

APPENDIX A.

TABLE A:1. Microorganisms reported isolated from root canals with necrotic pulps*.

STUDY	FAC. ANAEROBES	OBLIGATE ANAEROBES	OTHER
Möller (1966)	3,4,5,6,7	2,5,6,7,8,9,10,12,13	Candida, Sarcina
Bence *et al.* (1973)	NR	NR	NR
Berg & Nord (1973)	1,2,3,5,6,7	2,3,6,7,9,10,11,12	NR
Bergenholtz (1974)	3,5,6	2,5,7,8,10,12	Neisseria, Unident. Aerobes and Anaerobes
Kantz & Henry (1974)	1	2,4,6,7,9,11,12	Unident. Anaerobes
Wittgow & Sabiston (1975)	2,7,8	1,2,4,6,7,8,10,11,12	Unident. Anaerobes

*NR = not reported; Unident. = unidentified.

Key for Facultative anaerobes:

1 = *Actinomyces*
2 = *Staphylococcus*
3 = *Streptococcus* (alpha-hemolytic)
4 = *Streptococcus* (beta-hemolytic)
5 = *Streptococcus* (gamma-hemolytic)
6 = *Streptococcus* (enterococci)
7 = *Lactobacillus*
8 = *Undefined Streptococcus species*
9 = *Peptococcus*
10= *Peptostreptococcus*
11= *Propionibacterium*
12= *Veillonella*
13= *Leptotricia*

Obligate Anaerobes:

1 = *Arachnia*
2 = *Bacteroides*
3 = *Bifidobacterium*
4 = *Campylobacter*
5 = *Corynebacterium*
6 = *Eubacterium*
7 = *Fusobacterium*
8 = *Lactobacillus*
9 = *Peptococcus*
10= *Peptostreptococcus*
11= *Propionibacterium*
12= *Veillonella*
13= *Selenomas*

(continued)

STUDY	FAC. ANAEROBES	OBLIGATE ANAEROBES	OTHER
Keudell *et al.* (1976)	1,2,3,5	2,3,6,7,9,10,11,12	NR
Kaufman *et al.* (1976)	2,8	NR	*Candida,* Diptheroids, *Escherichia, Proteus*
Sundqvist (1976)	1	1,2,4,6,7,8,9,10,11,12,13	NR
Griffee NR *et al.* (1980)	2	NR	
Byström & Sundqvist (1981)	1	1,2,4,6,7,8,9,10,11,12	NR
Yoshida *et al.* (1987)	6,9	2,6,9,10,11,12	*Gafkya, Proteus*

* NR = not reported; Unident. = unidentified.

Key for Facultative anaerobes:
1 = *Actinomyces*
2 = *Staphylococcus*
3 = *Streptococcus* (alpha-hemolytic)
4 = *Streptococcus* (beta-hemolytic)
5 = *Streptococcus* (gamma-hemolytic)
6 = *Streptococcus* (enterococci)
7 = *Lactobacillus*
8 = *Undefined Streptococcus species*
9 = *Peptococcus*
10= *Peptostreptococcus*
11= *Propionibacterium*
12= *Veillonella*
13= *Leptotricia*

Obligate Anaerobes:
1 = *Arachnia*
2 = *Bacteroides*
3 = *Bifidobacterium*
4 = *Campylobacter*
5 = *Corynebacterium*
6 = *Eubacterium*
7 = *Fusobacterium*
8 = *Lactobacillus*
9 = *Peptococcus*
10= *Peptostreptococcus*
11= *Propionibacterium*
12= *Veillonella*
13= *Selenomas*

APPENDIX B. INFORMED CONSENT FORM

Bacteriology of Root Canals of Patients with (or without) Post-instrumentation Pain during Endodontic Treatment

I understand that I am being asked to participate in a study conducted by Drs. Safavi, Tanzer and Carrington, into the reasons why some patients experience painful and sensitive teeth after instrumentation for root canal treatment. I am being asked, upon coming to the clinic with such a sore tooth, to allow the sampling of the root canal in order to take a special culture of the root canal contents. This will hopefully help the doctors determine whether there are bacteria still in the root canal and if so, what bacterial types there are, and whether they may have caused my painful tooth.This procedure will take about 15 minutes and will cause no pain because my tooth will be anesthetized. The procedure will be quite similar to the one already done to culture my tooth. The procedure will not interfere in any way with the treatment of my tooth which will immediately continue after the bacterial sample is taken for the study.I understand that I am free to decline to participate in this study without prejudice to my care at the University of Connecticut Health Center. I also understand that I can expect no direct benefit from consenting to participate in this study. It is hoped that the information gained will help in understanding why some patients get sore teeth after endodontic instrumentation. I understand that the doctors will make their best efforts to maintain all information about me confidential. Any publication or report of the results of the study will not identify me by name. I have the opportunity to ask any questions about the procedure and the purpose of the study.It is not the present policy of the University of Connecticut to compensate human subjects in the event the research results in physical injury except that in fulfilling its public responsibility, the University of Connecticut Health Center/John Dempsey Hospital will have available the facilities and professional attention to care for subjects who may suffer a physical injury as a result of participation in this project.In such an event, please contact Rose Marie Howes (679-3173), who can review the matter with you and identify other sources that may be available to provide you with further information as to how to proceed.

I, THE UNDERSIGNED, HAVE UNDERSTOOD THE ABOVE EXPLANATION AND GIVE MY CONSENT TO VOLUNTARY PARTICIPATION IN THIS STUDY.

DATE:_____

_____
SIGNATURE OF SUBJECT

_____
SIGNATURE OF WITNESS

_____
SIGNATURE OF SOLICITING DOCTOR

(continued)

	1IIA: *P. saccharolyticus.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus approx. 0.8 μm by 1.0 μm., forms chains in Heart Infusion Broth (HIB).
Colonial# Characteristics:	Pulvinate/convex, entire, 1-2 mm, opaque.No hemolysis on BAK agar. Isolated only on BAK.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 5.6.
Biochemical Characteristics:	Acid produced from: fructose, glucose and mannose. Acid not produced from: arabinose, cellobiose, inositol, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose and xylose. Positive reactions: catalase activity and nitrate reduction. Negative reactions: esculin hydrolysis, gelatin liquefaction, indole production, litmus milk curd production/hydrolysis, starch hydrolysis and urease activity.
An-Ident## Results:	Positive reactions: PRO, ARL, ALA, GLY, and CAT. Identification: unable to confirm

# Denotes surface contour, marginal edge, size in diameter, color, and additional characteristics after 7 days anaerobic incubation.
##Substrate degradation by enzymes (see Appendix G).

TABLE C:2. Patient history and bacteriological findings of SPIP #2.

Patient #2:	44-yr-old male, Caucasian.
Med. Hx.:	Hypertension.
Meds.:	Tenormin.
Allergies/ Drug Reactions:	None.
Dental Hx.*:	21 Oct 85, tooth #1.4 (asymptomatic) has no restoration or caries. PAR; EPT= no response, Perio= 333F, 323L. 13 May 86, RC instrumentation; I_2KI on cotton pellet placed in chamber; access opening sealed with IRM.
Dental Tx.:	15 May 86, presents with SPIP. All procedures performed according to protocol. 17 May 86, patient reported having no pain.
Culture Results:	No growth.

* See Table C:1 for definitions of acronyms.

TABLE C:3. Patient history and bacteriological findings of SPIP #3.

Patient #3:	22-yr-old female, Caucasian.
Med. Hx.:	Asthma until 1984; xerostomia.
Meds.:	None.
Allergies/ Drug Reactions:	None.
Dent. Hx.*:	22 Jul 85, tooth #1.5 (asymptomatic), deep recurrent caries under DO amalgam, no PAR. EPT= normal response; Perio= 312F, 323L. 22 Jan 86, caries excavation and pulpotomy #1.5. 21 May 86, completed RC instrumentation; $Ca(OH)_2$ placed in canals; sealed access with IRM.
Dent. Tx.:	22 May 86, SPIP #1.5. All procedures according to protocol. 24 May 86, patient reported no pain.
Culture Results:	No growth.

* See Table C:1 for descriptions of acronyms.

TABLE C:4. Patient history and bacteriological findings of SPIP #4.

Patient #4:	39-yr-old female, Caucasian.
Med Hx.:	Appendectomy and hernia repair in 1980; TMJ dysfunction associated with 2-3 headaches per week.
Meds.:	Ibuprofen.
Allergies/ Drug Reactions:	Hives associated with Cephalosporin use.
Dental Hx.*:	17 Apr 86, tooth #4.4 (asymptomatic) diagnosed as having periapical condensing osteitis and mesial caries. EPT= normal response; Perio= 434F, 424L. RC instrumentation, $Ca(OH)_2$, IRM seal.
Dental Tx.:	20 Apr 86, SPIP #4.4 (began 2 days earlier but patient unwilling to seek emergency treatment). All procedures performed according to protocol.
	24 May 86, patient reports having no pain.
Culture Results:	No growth.

* See Table C:1 for definitions of acronyms.

TABLE C:5. Patient history and bacteriological findings in SPIP #5.

Patient #5:	31-yr-old female, Caucasian.
Med. Hx.:	None.
Meds.:	None.
Allergies/ Drug Reactions:	None.
Dent. Hx.*:	01 May 86, tooth #4.6 (asymptomatic) with PAR and defective, extensive MO amalgam. EPT= no response; Perio= 323F, 434L. 28 May 86, RC instrumentation; thioglycollate culture obtained; $Ca(OH)_2$ in canal; IRM seal.
Dent. Tx.:	30 May 86, SPIP. All procedures were performed according to protocol. (Note: all culturing procedures were performed with the original amalgam in place on the mesial surface of the tooth, possibly precluding good rubber dam isolation.) 03 Jun 86, patient reports slight discomfort.
Culture** Results:	5IA: *Streptococcus anginosus.**** 5IB: *Streptococcus sanguis* II. 5IIA *Streptococcus anginosus.* 5IIB: *Bacteroides gingivalis.* 5IIC: *Streptococcus anginosus* (5IIC=5IA). 5IID: *Fusobacterium* species (lost after id). 5IIE: *Streptococcus sanguis* II (5IIE=5IB) 5IIIA:*Streptococcus sanguis* II (5IIIA=5IIE=5IB). 5IIIB:*Streptococcus anginosus* (5IIIB=5IIC=5IA).

* See Table C:1 for definitions of acronyms.

** (See Coykendall, et al., 1987).

***By obtaining 3 different cultures (using 2 different culture media during this procedure) it was possible to detect the same bacterial species from different media. Thus, the reason, for example, 5IA (thioglycollate culture of tooth surface), 5IIC (MPRS culture of the root canal contents), and 5IIIB (thioglycollate culture of the root canal contents) all likely having the same species isolated.

(continued)

	5IA, 5IIC, 5IIIB: *S. anginosus*.
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus approx. 0.6 by 0.6 μm, forms chains in HIB.
Colonial# Characteristics:	Convex, entire, 0.5-1.0 mm, translucent, alpha hemolysis on BAK. Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 4.8.
Biochemical Characteristics:	Acid produced from: fructose, glucose, lactose, maltose, mannose, salicin, sucrose and trehalose. Acid not produced from: arabinose, cellobiose, inositol, mannitol, melibiose, raffinose, rhamnose, sorbitol and xylose. Positive reactions: esculin hydrolysis, litmus milk curd production and starch hydrolysis. Negative reactions: catalase activity, gelatin liquefaction, indole production nitrate reduction and urease activity.
Rapid Strep##:	Positive reactions: VP, ESC, LAP, ADH, LAC, TRE and AMD. Identification: *S. anginosus*.

See Table C:1 for descriptions of colonial characteristics.
##See Appendix G for description of use.

(continued)

	5IB, 5IIE, 5IIIA: *Streptococcus sanguis* II.
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus, approx. 0.8 by 0.8 µm; forms chains in HIB.
Colonial Characteristics:	Convex, entire, 0.5-1.0 mm, translucent, alpha-hemolysis on BAK agar. Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 4.8.
Biochemical Characteristics:	Acid produced from: fructose, glucose, lactose, maltose, mannose, melibiose, raffinose and sucrose. Acid not produced from: arabinose, cellobiose, inositol, mannitol, rhamnose, salicin, sorbitol, trehalose and xylose. Positive reactions: litmus milk curd production and starch hydrolysis. Negative reactions: catalase activity, esculin hydrolysis, gelatin liquefaction, indole production, nitrate reduction and urease activity.
Rapid Strep:	Positive reactions: LAP, LAC, RAF, AMD. Identification: *S. sanguis* II.

(continued)

	5IIA: *Streptococcus anginosus.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus approx. 0.8 by 0.8 μm; forms chains in HIB.
Colonial Characteristics:	Pulvinate, entire, 1-2 mm, opaque, beta-hemolysis. Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 4.3.
Biochemical Characteristics:	Acid produced from: fructose, glucose, maltose, mannose, salicin, sucrose and trehalose. Acid not produced from: arabinose, cellobiose, inositol, lactose, mannitol, melibiose, raffinose, rhamnose, sorbitol and xylose. Positive reactions: litmus milk curd production. Negative reactions: catalase activity, esculin hydrolysis, gelatin liquefaction, indole production, nitrate reduction, starch hydrolysis and urease activity.
Rapid Strep:	Positive reactions: (VP)~,(HIP), LAP, ADH, TRE and GLY. Identification: *S. anginosus.*

~( ) = weak reaction.

(continued)

	5IIB: *Bacteroides gingivalis.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-negative rod, approx. 1.0 by 1.6 μm; forms coccobacillary morphology in HIB.
Colonial Characteristics:	Pulvinate, entire, 1-2 mm, light brown to black. Isolated only on BAK; beta-hemolysis.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.5.
Biochemical Characteristics:	Acid produced from: (cellobiose)~. Acid not produced from: arabinose, fructose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose,raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. Positive reactions: esculin hydrolysis, gelatin liquefaction, indole production and litmus milk hydrolysis. Negative reactions: catalase activity, nitrate reduction, starch hydrolysis and urease activity.
An-Ident:	Positive reactions: IND, NGS, PHS, ARL, and ALA. Identification: *B. gingivalis.*

~( ) = weak reaction.

TABLE C:6. Patient history and bacteriological findings of SPIP #6.

Patient #6:	22-yr-old female, Caucasian.
Med. Hx.:	Hypertension.
Meds.:	None.
Allergies/ Drug Reactions:	Penicillin.
Dent. Hx.*:	18 May 86, tooth #4.6 with PAR and caries on distal extending to the osseous crest. EPT= no response; PERIO= 434F, 444L. 12 Jun 86, RC instrumentation; thioglycollate culture; I_2KI placed in chamber; IRM used to seal access opening.
Dent. Tx.:	13 Jun 86, returns with SPIP. All procedures performed according to protocol. 15 Jun 86, patient reports as having no pain, but 4 consecutive positive culture results were obtained by student provider.
Culture Results	6I: No growth. 6IIA: *Streptococcus anginosus.* 6IIIA: *Streptococcus anginosus* (6IIA=6IIIA).

* See Table C:1 for definitions of acronyms.

(continued)

	6IIA, 6IIIA: *S. anginosus.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus, approx. 0.8 by 1.2 μm; forms chains in HIB.
Colonial* Characteristics:	Convex, entire, 1-2 mm, translucent, alpha-hemolysis on BAK agar. Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 5.2.
Biochemical Characteristics:	Acid produced from: cellobiose, fructose, glucose, lactose, maltose, mannose, melibiose, salicin, sucrose and trehalose. Acid not produced from: arabinose, inositol, mannitol, raffinose, rhamnose, sorbitol and xylose. Positive reactions: esculin hydrolysis and litmus milk curd production. Negative reactions: catalase activity, gelatin liquefaction, indole production, nitrate reduction, starch hydrolysis and urease activity.
Rapid Strep:	Positive reactions: VP, ESC, LAP, ADH, LAC and TRE. Identification: *S. anginosus.*

* See Table C:1 for descriptions of colonial characteristics.

TABLE C:7. Patient history and bacteriological findings of SPIP #7.

Patient #7:	66-yr-old male, Caucasian.
Med Hx.:	Hypertension.
Meds.:	Salutensin.
Allergies/ Drug Reactions:	None.
Dent. Hx.*:	24 Jun 86, tooth #1.3 (asymptomatic) with PAR. EPT= no response; PERIO= 322F, 333L. In ER, RC instrumentation initiated with radiographic evidence of working length file placed 1 mm beyond the apex. Draining pus from the canal for over 30 min noted by ER resident.$Ca(OH)_2$ placed in canal and Cavit (Premier Dental Products, Philadelphia, PA) used to seal the access.
Dent. Tx.:	26 Jun 86, returns with SPIP. All procedures were performed according to protocol. 29 Jun 86, patient reports to having no pain.
Culture Results:	No growth from any of the three cultures; however, Gram-stained smear revealed gram-positive rods.

* See Table C:1 for definitions of acronyms.

TABLE C:8. Patient history and bacteriological findings of SPIP #8.

Patient #8:	22-yr-old male, Black
Med. Hx.:	Asthma
Meds.:	Theo-Dur
Allergies/ Drug Reactions:	None
Dent. Hx.*:	One year previously, patient visited the ER clinic with pain associated with tooth #4.4.Pulpectomy was performed.25 Aug 86, presents with complaint that temporary filling in #4.4 lost. Diffuse PAR; EPT= no response; PERIO= 323F, 333L. RC instrumentation; purulent exudate noted by ER resident. $Ca(OH)_2$ in canal and access sealed with Cavit.
Dent. Tx.	27 Aug 86, presents with SPIP. All procedures performed according to protocol. 28 Aug 86, reports no pain.
Culture Results:	8I: No growth. 8IIA: *Streptococcus anginosus.* 8IIB: *Bacteroides gingivalis.* 8IIIA: *Streptococcus anginosus* (8IIA=8IIIA).

* See Table C:1 for definitions of acronyms.

(continued)

	8IIA, 8IIIA: *S. anginosus.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive coccus, approx. 1.0 by 1.2 μm; forms chains in HIB.
Colonial* Characteristics:	Pulvinate/convex, entire, 0.5-1.0 mm, opaque, alpha-hemolysis on BAK agar. Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 4.3.
Biochemical Characteristics:	Acid produced from: cellobiose, fructose, glucose, lactose, maltose, mannose, melibiose, salicin, sucrose and trehalose. Acid not produced from: arabinose, inositol, mannitol, raffinose, rhamnose, sorbitol and xylose. Positive reactions: esculin hydrolysis, litmus milk curd production, and starch hydrolysis. Negative reactions: catalase activity, gelatin liquefaction, indole production,nitrate reduction and urease activity.
Rapid Strep:	(VP)**, (HIP), ESC, PYRA, (B-GAL), LAP, ADH, LAC, TRE and AMD. Identification: *S. anginosus.*

* See Table C:1 for descriptions of colonial characteristics.
** ( )= weak reaction.

(continued)

	8IIB: *Bacteroides gingivalis.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-negative rod, approx. 0.8 by 2.4 μm; forms coccobacillary morphology in HIB. Colonial
Characteristics:	Pulvinate, entire, 1-2 mm, light brown to black. Isolated only on BAK; beta-hemolysis.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.5.
Biochemical Characteristics:	Acid produced from: (cellobiose)~ Acid not produced from: arabinose, fructose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. Positive reactions: esculin hydrolysis, gelatin liquefaction, indole production and litmus milk hydrolysis. Negative reactions: catalase activity, nitrate reduction, starch hydrolysis and urease activity.
An-Ident:	Positive reactions: IND, NGS, PHS, ARL, and ALA. Identification: *B. gingivalis.*

~( ) = weak reaction.

TABLE C:9. Patient history and bacteriological findings of SPIP #9.

Patient #9:	27-yr-old female, Black
Med Hx.:	None
Meds.:	Tylenol #3
Allergies/ Drug Reactions:	None
Dent. Hx.*:	24 Sep 86, presents with severe pain. Tooth #2.3, an response; PERIO=333F, 323L. Bridge removed to treat the tooth, but inadequate anesthesia during pulpectomy precluded complete instrumentation of canal. 27 Sep 86, returns comfortable, and further RC instrumentation performed. Confirmatory working length radiograph obtained indicating that a #40 Hedström file was approximately 1 mm beyond the apex. Thioglycollate culture; $Ca(OH)_2$ placed in canal; sealed the access with Cavit and recemented bridge with Temp Bond (Kerr, Rhomulus, MI).
Dent. Tx.:	9 Sep 86, returns with SPIP. All procedures performed according to protocol. 30 Sep 86, returns without any pain.
Culture Results:	No growth.

* See Table C:1 for definitions of acronyms.

TABLE C:10. Patient history and bacteriological findings of SPIP #10.

Patient #10:	36-yr-old female, Hispanic
Med. Hx.	Asthma, anemia, and frequent headaches
Meds.:	Theo-Dur
Allergies/ Drug Reactions:	Pollen
Dent. Hx.*:	03 Dec 86, presents with tooth #4.1 (asymptomatic) with diffuse PAR extending from #3.1 to #4.2. EPT= no response; PERIO= 333F, 323L. RC working length file placed approximately 1 mm beyond apex. Complete canal instrumentation; $Ca(OH)_2$; IRM used to seal the access opening
Dent. Tx.:	05 Dec 86, presents with SPIP. All procedures were performed according to protocol.
Culture Results:	No growth.

*See Table C:1 for definitions of acronyms.

TABLE C:11. Patient histories and bacteriological findings of NPIP #1-4.

Patient #1:	37-yr-old male, Caucasian
Med. Hx.:	None
Meds.:	None
Allergies/ Drug Reactions:	Hayfever
Dent. Hx.*:	22 Oct 86, presents for RCT #4.1 (asymptomatic) with PAR (1 cm diam.) EPT= no response; PERIO= 323F, 333L. Complete RC instrumentation with evidence of file placed 1 mm beyond apex. Thioglycollate culture; $Ca(OH)_2$ in canal; IRM seal.
Dent. Tx.:	24 OCT 86, presents with #4.1 still asymptomatic. Three cultures obtained.
Culture Results:	No growth.
Patient #2	27-yr-old female, Black (SPIP #9)
Med. Hx.:	None
Meds.:	Tylenol #3
Allergies/ Drug Reactions:	None
Dent. Hx.:	(See Table A:9)
Dent. Tx.:	29 OCT 86, presents with SPIP 31 OCT 86, returns without any pain; three cultures obtained.
Culture Results:	No growth.

* See Table C:1 for definitions of acronyms.

(continued)

Patient #3:	23-yr-old male, Black
Med. Hx:	None
Meds.:	None
Allergies/ Drug Reactions:	None
Dent. Hx.:	10 FEB 86, presents for initial RCT #1.1; Pos.control culture obtained (see NP#3).
Dent. Tx.:	13 FEB 86, presents for RC instrumentation and cultures (tooth is asymptomatic) obtained.
Culture Results:	No growth.
Patient #4:	21-yr-old male, Caucasian
Med. Hx.:	None
Meds.:	None
Allergies/ Drug Reactions:	None
Dent. Hx.:	12 JAN 86, presents for RCT #3.6 (asymptomatic) with PAR associated with mesial and distal root apices. EPT= no response. PERIO=0.8 by 0.8 μm 434F, 434L. RC instrumentation; I_2KI in canals; IRM seal.
Dent. Tx.:	14 JAN 86, returns with tooth asymptomatic; RC cultures obtained.
Culture Results:	No growth.

TABLE C:12. History and bacteriological findings of NP #1.

Patient #1:	37-yr-old male, Caucasian (NPIP #1)
Med Hx.:	None
Meds.:	None
Allergies:/ Drug Rxns:	Pollen
Dent. Hx.:	22 OCT 86 presents for RCT #4.1* (asymptomatic) with PAR (1 cm in diameter). EPT**= no response; PERIO= 323F, 333L.
Dent. Tx.:	22 OCT 86 3 cultures were obtained.
Culture*** Results:	PC 1I: No growth. PC 1IIA: *Streptococcus anginosus.* PC 1IIB: *Streptococcus species* (anaerobic) PC 1IIIA: *Streptococcus anginosus.*

* Tooth identification according to FDI designation.

** EPT = electric pulp tests--normal response, no response, of hyperactive response; PERIO = periodontal pocket probing depths (in mm) of the facial (F) and lingual (L) portions of the tooth. PAR = periapical radiolucency; and RC = root canal.

*** The results of 3 different cultures obtained from each patient were reported: first, using thioglycollate broth, a culture for checking the adequacy of the operative field dis- infection (termed "I" culture); second, using MPRS transport medium, a culture for sampling and transporting canal contents (termed "II" culture); and third, using another thioglycollate broth, a culture for sampling canal contents (termed "III" culture) by which 0.9% sterile saline was used as the sampling fluid, subsequently absorbed by sterile paper cones, and placed into thioglycollate medium (according to accepted clinic procedures).

(continued)

	PC 1IIA, 1IIIA: *S. anginosus*.
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive cocci approx. 0.8-1.0 μm; forms shortchains in HIB.
Colonial^ Characteristics:	Pulvinate, entire, 0.5- 1.0 mm, translucent, beta hemolysis on BAK agar.Isolated on BAK, CFAT and MS agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 4.5.
Biochemical Characteristics:	Acid produced from: cellobiose, fructose,glucose, maltose, mannose, salicin,sucrose and trehalose. Acid not produced from: arabinose,inositol, lactose, raffinose, rhamnose,sorbitol, mannitol, melibiose and xylose. Positive reactions: esculin hydrolysis and litmus milk curd production. Negative reactions: catalase activity,gelatin liquefaction, indole production,nitrate reduction, starch hydrolysis and urease activity.
Rapid Strep#	Positive reactions: VP, (ESC)~ PAL, LAP, ADH and TRE. Identification: S. anginosus.

^Denotes surface contour, marginal edge, size in diameter, color, and additional characteristics after 7 days anaerobic incubation.
#Substrate degradation by enzymes (see Appendix G).
~( ) = weak reaction.

(continued)

	PC 1IIB: *Streptococcus* species (anaerobic).
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive cocci approx. 0.8 by 0.8 µm; forms chains in HIB.
Colonial Characteristics:	Pulvinate, entire, 0.5-1.0 mm, translucent, no hemolysis on BAK. Isolated only on BAK.
Culture Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.4.
Biochemical Characteristics:	Acid produced from: none. Acid not produced from: arabinose,cellobiose, fructose, glucose,inòsitol,lactose, maltose, mannitol, mannose, melibiose, salicin, sucrose, sorbitol,raffinose, rhamnose, trehalose and xylose. Positive reaction: esculin hydrolysis. Negative reactions: catalase activity,gelatin liquefaction, indole production,litmus milk curd production or hydrolysis, nitrate reduction, starch hydrolysis and urease activity.
An-Ident##:	Positive reactions: PRO and GLY. Identification: unable to speciate.

Substrate degredation by enzymes (see Appendix G).

TABLE C:13. History and bacteriological findings of NP #2.

Patient #2:	36-yr-old female, Hispanic (SPIP #10)
Med. Hx.:	(See Table A:10)
Meds.:	
Allergies:	
Dent. Hx.:	03 DEC 86, presents with tooth #4.1 (asymptomatic) with PAR* 1 by 2 cm in diameter.
Dent. Tx.:	03 DEC 86, three cultures obtained.
Culture Results:	2I: No growth. 2IIA: *Streptococcus* species (anaerobic) 2IIB: *Bacteroides* species 2III: No growth. 2IIA: *Streptococcus* species (anaerobic)
Cellular Characteristics:	Nonmotile, nonsporeforming, irregular staining gram-positive coccus, approx. 1.0 by 1.4 μm.
Colonial** Characteristics:	Convex, entire, 0.5-1.0 mm, opaque.Isolated only on BAK.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.7.
Biochemical Characteristics:	Acid produced from: fructose, lactose,maltose, mannose, salicin and sucrose. No acid produced from: arabinose,cellobiose, glucose, inositol, mannitol,melibiose, raffinose, rhamnose, sorbitol,trehalose and xylose. Positive reaction: none.Negative reactions: catalase activity,esculin hydrolysis, indole production,litmus milk curd production or hydrolysis, nitrate production, starch hydrolysis and urease activity.
An-Ident***:	Positive reactions: BDG, INA, ARG, PRO,ARL, ALA, HIS and GLY. Identification: unable to establish species.

* See Table C:1 for definitions of acronyms.
** See Table C1 for description of colonial characteristics.
*** See Appendix G for description of An-Ident.

(continued)

	2IIB: *Bacteroides*-like species.
Cellular Characteristics:	Nonmotile, nonsporeforming gram-negative rod approx. 0.8 by 4.0 μm.
Colonial Characteristics:	Pulvinate, entire, 0.5-1.0 mm, brown, sticks to agar, beta-hemolysis on BAK.Isolated only on BAK.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.4.
Biochemical Characteristics:	Acid produced from: fructose and mannose. No acid produced from: arabinose, cellobiose, glucose, inositol, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sucrose, trehalose, sorbitol and xylose. Positive reactions: catalase activity & indole production. Negative reactions: esculin hydrolysis, gelatin liquefaction, litmus milk curd production or hydrolysis, nitrate reduction, starch hydrolysis and urease activity.
An-Ident:	Positive reactions: NGS, NPG, ARG, LEU, PRO, PYR, ARL, ALA, HIST, PHA, GLY and CAT Identification: unable to establish.

TABLE C:14. History and bacteriological findings of NP #3.

Patient #3:	23-yr-old male, Black (NC Patient #3)
Med. Hx.:	None
Meds.:	None
Allergies:	None
Dent. Hx.:	03 FEB 86, presents with complaint of constant, severe pain tooth #1.1 of 1 day's duration following trauma. Tooth had been traumatized 1 year earlier without symptoms. Clinical exam revealed intact tooth, buccal & palatal swelling, and PAR* 1 cm in diameter.
Dent. Tx.:	10 FEB 86, PC cultures were obtained.
Culture Results:	3I: No growth. 3IIA: *Actinomyces viscosus.* 3IIB: *Lactobacillus* sp. 3IIC: *Lactobacillus fructivorans.* 3IID: *Fusobacterium nucleatum.* 3IIIA: *Actinomyces viscosus* (3IIA= 3IIIA). 3IIIB: *Lactobacillus* sp. (3IIB= 3IIIB). 3IIIC: *Lactobacillus fructivorans* (3IIC=3IIIC). 3IIID: *Fusobacterium nucleatum* (3IID= 3IIID).

* See Table C:1 for definitions of acronyms.

(continued)

	3IIA,3IIIA: *A. viscosus.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive rod approx. 0.8 by 2.4 μm.
Colonial# Characteristics:	Convex, entire, < 0.5 mm, translucent. Isolated on BAK and CFAT agars.
Cultural Characteristics:	Facultative anaerobe; terminal pH in glucose-containing HIB is 5.4.
Biochemical Characteristics:	Acid produced from: fructose, glucose, lactose, maltose, mannose, raffinose and sucrose. No acid produced from: arabinose, cellobiose, inositol, mannitol, melibiose, rhamnose, salicin, sorbitol, trehalose and xylose.Positive reactions: catalase activity, esculin hydrolysis and nitrate reduction. Negative reactions: gelatin liquefaction, indole production, litmus milk curd production, starch hydrolysis, and urease activity.
An-Ident##:	Positive reactions: ADG, BDG, PHS, GAL, INA, LEU, PRO, TYR, ARL, ALA, HIST, PHA, GLY and CAT. Identification: *A. viscosus.*

See Table C:1 for descriptions of colonial characteristics.
##See Appendix G for descriptions.

(continued)

	3IIB, 3IIIB: Lactobacillus-like species.
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive rod approx. 0.8 by 2.4 μm.
Colonial Characteristics:	Convex, entire, << 0.5 mm, translucent. Isolated on BAK & Rogosa SL agars.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.7.
Biochemical Characteristics:	Acid produced from: fructose. No acid produced from: arabinose, cellobiose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sucrose, sorbitol, trehalose and xylose. Positive reactions: none. Negative reactions: catalase activity, esculin hydrolysis, gelatin liquefaction, indole production, litmus milk curd production, nitrate reduction, and urease activity.
An-Ident:	Positive reactions: none. Identification: unable to confirm.

(continued)

	3IIC, 3IIIC: *L. fructivorans.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-positive rod approx. 1.5 by 4.0 µm.
Colonial Characteristics:	Convex, entire, <0.5 mm, translucent with speckled center, odor. Isolated on BAK and Rogosa SL agars.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 6.7. Forms chains in HIB.
Biochemical Characteristics:	Acid produced from: fructose. No acid produced from: arabinose, cellobiose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sucrose, sorbitol, trehalose and xylose. Positive reaction: none. Negative reactions: catalase activity, esculin hydrolysis, gelatin liquefaction, indole production, litmus milk curd production, or hydrolysis, nitrate reduction, starch hydrolysis, and urease activity.
An-Ident:	Positive reactions: PHS and INA. Identification: unable to confirm.

(continued)

	3IID, 3IIID: *F. nucleatum.*
Cellular Characteristics:	Nonmotile, nonsporeforming, gram-negative rod approx. 0.6 by 8.0 µm.
Colonial Characteristics:	Umbilicate, irregular margins, 1-2 mm, opaque, looks like "molar tooth". Isolated on BAK and FEA agars.
Cultural Characteristics:	Obligate anaerobe; terminal pH in glucose-containing HIB is 5.2.
Biochemical Characteristics:	Acid produced from: fructose and glucose. No acid produced from: arabinose, cellobiose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. Positive reaction: indole production. Negative reactions: catalase activity, esculin hydrolysis, gelatin liquefaction, litmus milk curd production or hydrolysis, nitrate reduction, starch hydrolysis and urease activity.
An-Ident:	Positive reaction: INA. Identification: *F. nucleatum.*.

TABLE C:15. History and bacteriological findings of NP #4.

Patient #4:	19-yr-old male, Caucasian
Med. Hx.:	None
Meds.:	None
Allergies:	None
Dent. Hx.:	One year previously patient received trauma to anterior teeth. No symptoms, PAR*, or caries.EPT= no response; PERIO= 222F, 323L.
Dent. Tx.:	15 MAY 87, 3 cultures obtained.
Culture Results:	4I: No growth. 4IIA: No growth. 4IIIA: No growth.

* See Table C:1 for definitions of acronyms.

APPENDIX D. FORMULAE FOR BACTERIOLOGICAL AGARS

BAK:

Demineralized H_2O.....949.0ml
Brucella Agar (BBL).....43.0g
Hemin (Sigma).....0.005g (5µg/ml)
Sheep Blood (Colorado Serum Co.,
Denver, CO).....50.0ml
Menadione (Sigma).....0.010g (10µg/ml)

1. Add Brucella & 1 ml of Hemin solution (5mg/ml: 0.5g of hemin in 10 ml 1 N NaOH, up to 100 ml with dist. H_2O)
2. Adjust pH to 7.0 (1 M NaOH); heat with agitation & boil 1 minute.
3. Autoclave 121° C, 15 min; place in 50° C water bath for 1 hour.
4. Add 1 ml of filtered (0.22 µm pore size, Millipore Corporation, MA) menadione solution (dissolved in dist. H_2O) and Sheep's blood.
5. Dispense, store 1/2 in refrigerator, 1/4 in chamber, 1/4 for candle jar use.

BBE:

Demineralized H_2O.....1000.0ml
Trypticase Soy Agar (BBL).....40.0g
Oxgall (Difco).....20.0g
Esculin (Difco).....1.0g
Ferric Ammonium Citrate (Fischer
Scientific Co., Pittsburgh, PA).....0.5g
Hemin (Sigma).....5.0mg/ml
Gentamicin (Sigma) 40.0mg/ml

1. Dissolve T Soy, Oxgall Esculin and Ferric Ammonium Citrate in dist. H_2O
2. Add 2.0 ml of Hemin solution.
3. Add 2.5 ml of Gentamicin solution (this was omitted).
4. Adjust pH to 7.0 with 1 M NaOH.
5. Autoclave @ 121° C for 15 minutes.
6. Dispense into petri dishes, place in chamber.

CFAT:

Demineralized H2O 850.0ml
T Soy Broth (BBL).....30.0g
Agar (BBL).....15.0g
Cadmium Sulfate (Sigma).....0.013g (in 50 ml d. H_2O)
Sodium Fluoride (J.T. Baker Chemical Co., Phillipsburg, N.J) .....0.08g ""
Neutral Acriflavin (Sigma).....0.0012g ""
Basic Fuchsin (Sigma).....0.00025g ""
Glucose (Baker) 5.00g
Potassium Tellurite (Difco).....0.25ml 1%
Chapman's Sheep Blood (Colorado Serum).....50.0ml

1. Add T Soy Broth & Agar to dist. H_2O
2. Add CS, SF, Na, and BF (in 50 ml sol'n).
3. Add Glucose; adjust pH to 7.3 (1 M NaOH).
4. Autoclave @ 121° C, 15 min., cool to 50° C in water bath.
5. Add Tellurite & Sheep's blood.
6. Dispense, use in candle jar.

Clindamycin, THFF-KNO_3:

Demineralized H_2O 1000.0ml
Todd Hewitt Broth (BBL).....30.0g
Agar (BBL).....15.0g
KNO_3 (Bakers').....2.0g
Hemin (Sigma).....0.005g
Clindamycin (Sigma).....0.005g/ml (5 µg/ml)

1. Add ingredients except Clindamycin.
2. Autoclave @ 121° C for 15 min.; cool to 50° C.
3. Add filtered (0.22 µm) Clindamycin.
4. Pour plates; for candle jar use.

EMB (Difco):

Demineralized H_2O.....1000.0ml
Bacto-Peptone 10.0g
Bacto-Lactose 5.0g
Bacto-Saccharose 5.0g
K2HPO4 .....2.0g
Agar 13.5g
Eosin Y 0.4g
Methylene Blue 0.065g

1. Add 36g of dehydrated EMB to dist. H2O; adjust pH to 7.1.
2. Autoclave @ 121° C for 15 min; cool to 50° C; dispense for candle jar use.

FEA:

Demineralized H_2O 975.0ml
Brucella Agar (BBL).....43.0g
Na_2HPO_4 (Baker).....5.0g
KH_2PO_4 (Baker).....1.0g
$MgSO_4$ (Baker).....0.1g
Hemin (Sigma).....0.005g
Polysorbate 80 (Sigma).....1.0ml
Vancomycin (Sigma).....0.10g/ml
Erythromycin (Sigma).....0.003g/ml
Egg Yolk (store-bought, medium size) 25.0ml (2.5% vol/vol)

1. Add ingredients except antibiotics; adjust pH to 7.6 (1 M NaOH).
2. Autoclave @ 121° C for 15 min.; cool to 50° C.
3. Mix equal parts Egg Yolk (remove aseptically from shell) to dist. H_2O up to 25 ml; add to medium.
4. Add 1 ml each (dissolved in dist. H_2O) of antibiotic sol'n.
5. Pour into petri dishes; use in anaerobic chamber.

GSTB:

Demineralized H_2O 800.0ml
Sol'n A:
Trypticase Peptone (BBL).....5.0g
Yeast Extract (Difco).....5.0g
K_2HPO_4 (Baker).....5.0g
Na_2CO_3 (Baker).....0.05g
Jordan's salt sol'n:
1.15g of $MgSO_4$, 0.19g of $MnSO_4$,
0.068g of $FeSO_4$ per 10 ml Dist. H_2O.....0.5ml
Agar (BBL).....20.0g
Sol'n B:
Sucrose (Baker).....50.0g (100 ml d. H_2O)
Sol'n C:
Glucose (Baker).....50.0g (100 ml d. H_2O)
Potassium Tellurite (Difco).....1.0ml (1% sol'n)
Bacitracin (Difco).....1.0ml (0.44g/ml)

1. Add Sol'n A to dist. H_2O; adjust pH to 7.2 (1 M HCl).
2. Add agar and boil for 1 minute.
3. Autoclave Solutions A, B, C (separately) @ 121° C for 15 min; mix when cooled to 50°C.
4. Add filtered (0.22 μm) Chapman's & Bacitracin.
5. Dispense; use in candle jar.

Rogosa SL Agar:

Demineralized H_2O.....1000.0ml
Bacto-Tryptone.....10.0g
Bacto-Yeast Extract.....5.0g
Bacto-Dextrose.....10.0g
Bacto-Arabinose.....5.0g
Bacto-Saccharose.....5.0g
Sodium Acetate.....15.0g
KH_2PO_4.....6.0g
Ammonium Citrate.....2.0g
$MgSO_4$.....0.57g
$MnSO_4$.....0.12g
$FeSO_4$.....0.03g
Sorbitan Monooleate.....1.0g
Bacto-Agar.....15.0g
Acetic Acid 1.32ml
1. Dissolve 75 grams of dehydrated material into dist. H_2O.
2. Add Acetic Acid; dispense into plates; use in chamber.

MS (Difco):

Demineralized H_2O 1000.0ml
MS Agar 90.0g
Bacto-Chapman's sol'n (Difco).....1.0ml

1. Boil MS agar in dist. H_2O for 1 min.; autoclave @ 121° C for 15 minutes.
2. Add filtered (0.45 µm) Bacto-Chapman's sol'n aseptically.
3. Dispense; use for candle jar.

Mannitol-Salt (Difco):

Demineralized H_2O 1000.0ml
Proteose Peptone #3.....10.0g
Bacto-Beef Extract.....1.0g
D-Mannitol.....10.0g
NaCl.....75.0g
Bacto-Agar.....15.0g
Phenol Red.....0.025g

1. Dissolve 111g of ingredients in dist. H_2O; final pH 7.4.
2. Autoclave @ 121° C for 15 min.; cool to 50° C; dispense for candle jar use.

TSBV (1/2 Liter):

Demineralized H_2O 425.0ml
T Soy Agar (BBL, Cockeysville, MD).....20.0g
Yeast Extract (Difco, Detroit, MI).....0.5g
Horse Serum (Sigma Chemical Co.,
St. Louis, MO).....50.0ml
Bacitracin (Difco).....0.0375g (into 25 ml H_2O)
Vancomycin (Sigma).....0.0025g

1. Add T Soy & Yeast Extract to dist. H_2O.
2. Adjust pH to 7.2, using 1 M NaOH; autoclave @ 121° C for 15 minutes; place in 50° C water bath for 1 hour.
3. Add 50 ml filtered, sterilized horse serum (after heat-inactivated by 56°C H_2O water bath for 30 min.).
4. Add bacitracin & vancomycin (filter sterilized, 0.22-µm pore size) to 25 ml dist. H_2O
5. Dispense in petri dishes (good for up to 10 days) for use in chamber.

APPENDIX E. FORMULAE FOR BACTERIOLOGICAL BROTHS

Heart Infusion Broth (Difco)

Demineralized H_2O.....900.0ml
Beef Heart Muscle, Infusion from
Tryptose or Thiotone Peptic Digest
of Animal Tissues USP 375.0g
NaCl.....5.0g
0.6% Solution of Carbohydrate.....100.0ml

1. Mix 22.5g of dehydrated material into dist. H_2O; autoclave @ 121° C for 15 minutes.
2. Add carbohydrate by filter sterilization (0.22 µm).
3. Dispense 5-10 ml into sterile 13- by 100-mm test tubes.

Indole-nitrate Broth

Demineralized H_2O 1000.0ml
Tryptone
Yeast Extract.....3.0g
Potassium Nitrate.....2.0g

Nitrate Reagent A:
a-Naphthylamine.....5.0g
5 N Acetic Acid.....1000.0ml

Nitrate Reagent B:
Sulfanilic Acid.....8.0g
5 N Acetic Acid.....1000.0ml

1. Dissolve ingredients by boiling for 1 min. and adjust pH to 7.2 + 0.1.
2. Autoclave @ 121° C for 15 min.
3. Test for indole production by adding 1 drop of Kovac's reagent to 24 hr inoculated strain; a pos. reaction is red.
4. Test for nitrate reduction by adding 1 drop of nitrate reagent A and 1 of B; a pos. reaction is red.

Nutrient Gelatin

Demineralized H_2O 1000.0ml
Beef Extract (BBL) 3.0g
Peptone (Difco).....5.0g
Gelatin (Difco) 120.0g

1. Add ingredients to distilled H_2O; pH 6.8.
2. Dispense 5 ml into 13 x 100 mm test tubes & autoclave @ 121° C for 5 min.; store in refrigerator.
3. Inoculate by stabbing into solid medium a depth of 1/2 to 1 inch. Set an uninoculated control tube to be run along with the bacterium being tested.
4. Incubate at 35° C & test daily for liquefaction of gelatin.

Trypticase Soy Broth with 0.04% Resazurin (To Test Anaerobiosis)

Demineralized H_2O.....500.0ml
Glucose 5.0g
Resazurin 2.0g

1. Mix ingredients and store in anaerobic chamber.

Urease Broth (Stuart's)

Demineralized H_2O.....1000.0ml
Yeast Extract (Difco).....0.1g
KPO_4 (Baker).....9.1g
Na_2PO_4 (Baker)..... 9.5g
Urea (Sigma).....20.0g
Phenol Red (Sigma).....0.01g

1. Dissolve ingredients in dist. H_2O & filter (0.22 μm) 2 ml into sterile test tubes.
2. Inoculate broth with loopful of pure culture; use positive and negative controls.
3. Watch for red color which indicates alkalinization and therefore urea hydrolysis.

APPENDIX F.

TABLE F:1. Formulae and preparations of 3 transport media.

MPRS		RTF		VMG III	
NaCl	2.25g	NaCl	0.90g	NaCl	1.00g
KCl	0.11g	K_2HPO_4	0.45g	KCl	0.42g
$CaCl_2$	0.63g	KH_2PO_4	0.45g	$CaCl_2$	0.24g
		MgSO4	0.19g	$MgSO_4$	0.10g
		$(NH_4)_2SO_4$	0.90g		
		Na_2CO_3	0.40g		
		EDTA	0.1M		
SODIUM META-PHOSPHATE	10.00g			PHENYL-MERCURIC ACETATE	0.003g
				SODIUM GYLCERO-PHOSPHATE	10.00g
RESAZURIN	1µg	RESAZURIN	1µg	METHYLENE BLUE	0.002g
				AGAR (NOBLE)	2.00g
				BACTO-GELATIN	0.00g
				TRYPTOSE	0.50g
				THIOTONE	0.50g
L-CYSTEINE	0.50g	DTT	0.20g	L-CYSTEINE	0.50g
				THIOGLYCOLLIC ACID	0.50g
DEIONIZED H_2O	1000 ml	DEIONIZED H_2O	1000 ml	DEIONIZED H_2O	1000 ml

MPRS:

1. Add salts to demin. H_2O; mix DTT, $NaPO_3O_9$ & Cysteine in 25 ml of demin. H_2O & add to salt solution. Adjust pH to 7.0 and filter (0.22 µm) before storing in chamber.

RTF:

1. Add salts to demin H_2O; mix DTT & $NaCO_3$ in 25 ml of demin. H_2O & add to salt solution. Filter (0.22 µm) and store in chamber; the pH is 8.2 before placing in the chamber but will drop to 7.0 after 24-48 hr.

VMG III:

1. Dissolve Bacto-gelatin, Tryptose, Thiotone, Cysteine Hydrochloride, and Thioglycollic Acid into 900 ml solution containing dissolved Agar (by heat) after this solution has cooled to about 50° C.

2. Add remaining ingredients to 100 ml of distilled water and then add this to the 900 ml solution, adjusting pH to 7.5 with NaOH.

3. Dispense into 5 ml screw-capped vials (to the brim) and autoclave @ 212 C for 15 min; store in chamber.

APPENDIX G. DESCRIPTION OF RAPID IDENTIFICATION STRIPS

An-Ident System:

This system is designed for *in vitro* identification of anaerobes using 20 miniaturized conventional and chromogenic substrates. Using the manufacturer's instructions the following substrates are tested: indole (IND), P*-N-acetyl-b-D-glucosaminide(NGS), P-a-D glucoside (ADG), P-a-L-arabinofuranoside (ARB), P-b-D glucoside (BDG), P-a-L-fucoside (FUC), P-phosphate (PHS), P-a-D-galactoside (GAL), P-b-D- galactoside (NPG), indoxyl-acetate (INA), arginine (ARG),L- leucyl-4-methoxy-B** (LEU), L-proline-B (PRO), L-pyrrolidonyl-B (PYR), L-tyrosine-B (TYR), L-arginine-B (ARL), L-alanyl-L-alanyl-B (ALA), L-histidine-B (HIS), L-phenyl- alanine-B (PHA), and L-glycine-B (GLY). A seven-digit profile is derived from the results (after 4 hr aerobic incubation) from the clinical isolates and is compared to the Analytical Profile Index derived from isolates studied in the API Anaerobe Laboratory. Twenty biochemical tests are divided into 6 groups of 3 and a 7th group of 2.

Rapid Strep:

A 7-digit profile number is derived from the results (after 4-24 hr aerobic incubation) from the clinical isolates and is compared with a similar profile in the computer-stored data base. The following substrates are evaluated: acetoin production (VP), hippurate hydrolysis (HIP), esculin (ESC), pyrrolidonylarylamidase (PYRA), a-galactosidase (aGAL), B-glucuronidase (BGUR), B-galactoidase (BGAL), alkaline phosphatase (PAL), leucine arlylmidase (LEU), arginine dehydrolase (ADH), ribose (RIB), arabinose (ARA), mannitol (MAN), sorbitol (SOR), lactose (LAC), trehalose (TRE), inulin (INU), raffinose (RAF), starch (AMD) and glycogen (GLYG). Also, the last entry designates the presence of absence of beta- hemolysis. Esculin hydrolysis and all of the carbohydrates are evaluated up to 24 hr whereas the other substrates are recorded after 4 hr incubation.The profile number generated is given a "very good" to "low discrimination" value and additional tests as needed are performed.

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*P = p-nitrophenol
**B = B-naphythlamide
a = alpha; B = beta.

APPENDIX H. LIST OF ABBREVIATIONS

ANO_2.....Anaerobic
API.....Analytab Products Incorporated
BAK.....Blood Agar with Vitamin K
BBE.....Bacteroides, Bile, Esculin (agar)
BHIA.....Brain Heart Infusion Agar
Biochem.....Biochemical
Carb.....Carbohydrate
CFAT.....Columbia Fuchsin Acridine Tellurite
CM.....Chopped Meat
CMG.....Chopped Meat Glucose
Dist.....Distilled
DO.....Disto-occlusal
DTT.....Dithiotreitol
EDTA.....Ethylenediaminetetraacetic Acid
EMB.....Eosin Methylene Blue (agar)
EPT.....Electric Pulp Test
F.....Facial
FAC.....Facultative
FDI.....Federation Dentaire International
FEA.....Fusobacterium Egg Agar
GSTB.....Glucose, Sucrose, Tellurite, Bacitracin, (agar)
HEM.....Hemolysis
HIB.....Heart Infusion Broth
Hydrol.....Hydrolysis
IRM.....Intermediate Restorative Material
L.....Lingual
MFG.....Manufacturer
MO.....Mesio-occlusal
MPRS.....Modified, Prereduced Ringer's Solution
MS.....Mitis Salivarius (agar)
NP.....Necrotic Pulp
NPIP.....No Post-Instrumentation Pain
OB.....Obligate
PAR.....Periapical Radiolucency
Perio.....Periodontal
PCT.....Paper Cone Transport
PY.....Peptone Yeast
PYG.....Peptone Yeast-Glucose
RC.....Root Canal
RCT.....Root Canal Therapy
RTF.....Reduced Transport Fluid
RXNS.....Reactions
SPIP.....Severe Post-Instrumentation Pain
TMJ.....Temporo-Mandibular Joint
TSBV.....Tripticase Soy Bacitracin Vancomycin (agar)
VMG.....Viability-Preserving Media
WL.....Working Length

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